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Free amino acid, protein and water content changes associated with seed development in *Araucaria angustifolia*

L.V. ASTARITA, E.I.S. FLOH and W. HANDRO*

Plant Cell Biology Laboratory, Department of Botany, Institute of Biosciences, University of São Paulo, IB-USP, CP 11461, 05422-970, São Paulo, Brazil

Abstract

The free amino acid, protein, water and dry matter contents were determined during the seed development of *Araucaria angustifolia*. Soluble and insoluble proteins in the mature seed represent 4.2 % of the fresh matter. The embryonic axis stored the greatest amount of soluble proteins, while cotyledons both with the embryonic axis showed the largest quantities of insoluble proteins in the mature seed. The greatest concentration of free amino acids was detected during the stage when cotyledons start to develop. Glutamic acid, aspartic acid, alanine and serine were predominant in the whole seed while arginine, lysine and γ-aminobutyric acid were present in great amounts only in cotyledons and embryonic axis. Although megagametophyte was important as a source of free amino acids, it was not the major protein storage organ in the mature seed. In the embryogenetic process, the rise of cotyledons is closely related to physiological and biochemical changes.

Additional key words: Brazilian pine, zygotic embryogenesis.

Introduction

The conifer *Araucaria angustifolia* (Bert.) O. Kuntze (Brazilian pine), grows naturally in Brazil and is the only native gymnosperm of economic importance. Evolutionary studies on *Araucaria* suggested that this genus is the most primitive amongst the conifers, showing an embryogenetic pattern which differs from that known for other conifers such as *Pinus, Taxus* and *Sequoia* (Haines and Prakash 1980, Gifford and Foster 1989). There are few studies concerning the physiological and biochemical aspects of embryo development in *Araucaria* (Owens *et al.* 1997). This lack of knowledge on zygotic embryology presents a barrier to improving *in vitro* somatic embryogenesis as an alternative for reforestation programs (Astarita and Guerra 1998) and does not allow for comparative studies with other conifers.

Despite the use of mature zygotic embryos for obtaining somatic embryos in *Picea* species (Egertsdotter and Von Arnold 1998), up to now it has been extremely difficult to induce somatic embryogenesis in *Pinus* and *Araucaria* zygotic embryos (Bozhkov *et al.* 1997, Guerra *et al.* 2000). Previous studies on somatic embryogenesis in *A. angustifolia* indicated that the ability of pre-cotyledonary zygotic embryos to produce embryogenic cultures is restricted to a few months, ceasing when cotyledons development starts (Astarita and Guerra 1998).

Storage proteins are synthesized in large amounts in specific tissues during certain stages of seed development. Developing seeds maintain nitrogen reserves as storage proteins (Müntz *et al.* 1998). Mature gymnosperm seeds store nutritive macromolecules in the megagametophyte, sources of monosacharides and amino acids for embryonic axis development during germination (Groome *et al.* 1991). On the other hand, the pattern of amino acid

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* Corresponding author; fax: (+55) 11 30917547, e-mail: wthandro@usp.br

flux in zygotic embryos could serve as a model reference for somatic cell cultures of conifers, as envisaged by Durzan and Durzan (1991).

In this context, this paper characterizes the physiological and biochemical changes occurring during particular stages of seed development in *A. angustifolia*, which are useful in further studies to understand the events associated with the responsive period of somatic embryo induction.

Material and methods

Plants: Female cones of *A. angustifolia* were harvested in the summer, from December to March, in a natural population (Campos de Jordão State Park, São Paulo, Brazil). Harvest dates corresponded to the sequential development stages from which samples were taken for biochemical analysis (stages 1 to 6, Fig. 1*a-d*). The last harvest date was defined as the phase of development when the seeds were able to complete germination (stage 6). At this stage, the megagametophyte represents 97 - 98 % of the seed fresh matter.

Harvest dates were used to identify different stages of seed development. Samples harvested in stage 1 (Fig. 1*a*) showed an immature embryo, mucilaginous megagametophyte and a suspensor attached to the embryonic axis, while after stage 3, the embryo was organized with a primordial cotyledon structure (Fig. 1*a,b*). Although small cotyledons were present in stage 3, they were only extractable following stage 4. Immature and mature seeds were removed from the cones and dissected to separate the gametophyte, embryonic axis and cotyledon tissues when present. Tissue samples were stored at -20 °C before biochemical analysis.

Amino acids: Tissue samples were frozen and homogenized with 10 cm^3 80 % ethanol three times, and concentrated under a nitrogen flow. The samples were re-suspended in 3 cm^3 water and centrifuged at 20 000 *g* for 2 min. The supernatant was passed through a mini-column Sep-Pak C_{18} (*Waters*), previously conditioned with 35 % aqueous methanol, and sequentially eluted with 1 cm^3 35 % and 65 % methanol. Amino acids were derivatizated with *o*-phthaldialdehyde (OPA) as previously described (Marur *et al.* 1994). Aliquots of 0.1 cm^3 were derivatizated with 0.3 cm^3 OPA solution during 10 s before injection. The amino acids in each sample were analyzed by high-performance liquid chromatography (HPLC) on a C_{18} reverse-phase column (*Shimadzu Shim-pack CLC ODS*). The solvent 1: 65 % methanol, and solvent 2: a solution of 50 mM sodium acetate, 50 mM sodium phosphate, 1.5 cm^3 m^{-3} acetic acid, 20 cm^3 m^{-3} methanol and 20 cm^3 m^{-3} tetrahydrofuran, pH 7.25, were combined in the following gradient based on the percent of solvent 2: 35 - 40 % (0.01 - 18 min), 40 - 65 % (18 - 24 min), 65 - 68 % (24 - 35 min) and 68 - 100 % (35 - 45 min), at a flow rate of 1 cm^3 min^{-1}. Fluorescence excitation and emission wavelength of 250 and 480 nm, respectively, was used for amino acid detection. Peak areas and retention times were measured by comparison with known quantities of standard amino acids: alanine (Ala), aspartic acid (Asp), glutamic acid, asparagine, serine (Ser), arginine, glutamine (Glu), histidine,

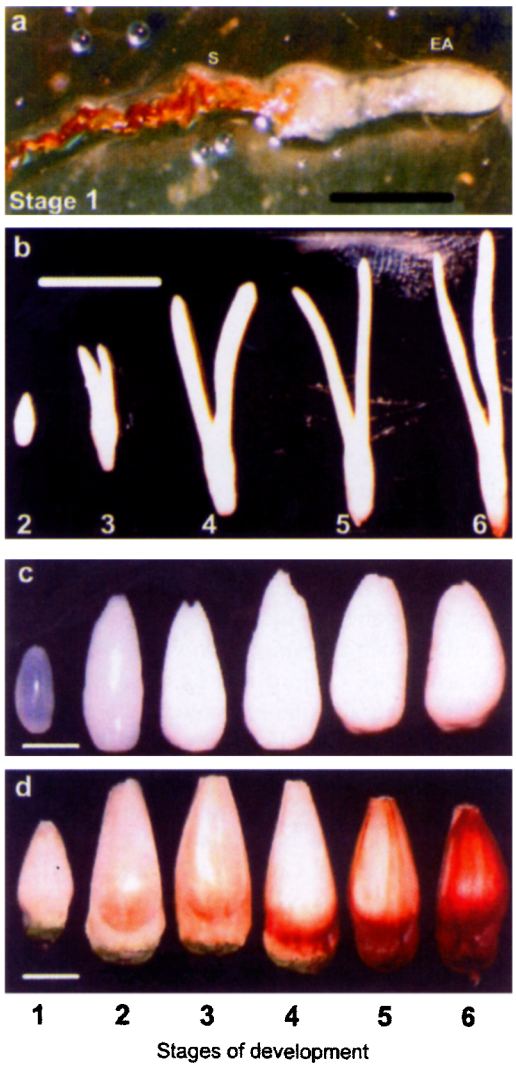

Fig. 1. Stages of embryonic axis development of *A. angustifolia* seeds collected on different dates. Stage 1 (10 Dec.), 2 (10 Jan.), 3 (26 Jan.), 4 (11 Feb.), 5 (4 Mar.) and 6 (25 Mar.). *a*: immature embryo (s - suspensor, AE - embryonic apex); *b*: stages of embryo development; *c*: megagametophyte stages; *d*: seed stages. *Bar* (*a*) represents 0.5 cm, and *bars* (*b,c,d*) represent 1 cm.

γ-aminobutyric acid (GABA), glycine, threonine, alanine, tyrosine, methionine, tryptophan, valine, phenylalanine, isoleucine, leucine, lysine and ornithine.

Dry matter and water content: Fresh matter was determined in the gametophyte, embryonic axis and cotyledon tissues when present. The different materials were maintained for 72 h at 110 °C, to obtain the dry matter. Water content was reported as a percentage of dry matter.

Protein determination: Seed tissues (0.5 g fresh matter) were homogenized in 2 cm^3 of 0.05 M Na-phosphate buffer (pH 7.5) with 10 mM β-mercaptoethanol, at 4 °C. After 30 min, the homogenates were centrifuged at 40 000 *g* for 30 min at 4 °C, and re-extracted three times. The supernatants containing the soluble protein fraction from the cytoplasm and the crystalloid matrix were measured. The pellets were re-suspended and solubilized by boiling (5 min) in an extraction buffer containing Tris-HCl 65 mM (pH 6.8), 2 % SDS, 10 % glycerol and 2.5 % β-mercaptoethanol and centrifuged at 40 000 *g* for 30 min. The pellet was re-extracted three times. The supernatant containing the crystalloid proteins (insoluble fraction) was used for determination (Becker *et al.* 1978, Gifford *et al.* 1982, Gifford 1988, Lammer and Gifford 1989). The protein content was determined according to Bradford (1976) using bovine serum albumin as a standard.

Statistics: Two different aliquots were used for each sample (10 seeds), and three determinations were made for each aliquot. The reported data are averages of convergent repetitions. The vertical bars in the graphs represent standard errors.

Results and discussion

Water content: The water content changes observed for *A. angustifolia* seed tissues (Fig. 2*a*) were typical of recalcitrant seeds, which remain viable due a relatively high moisture content. The water content of the embryonic axis and megagametophyte rapidly increased

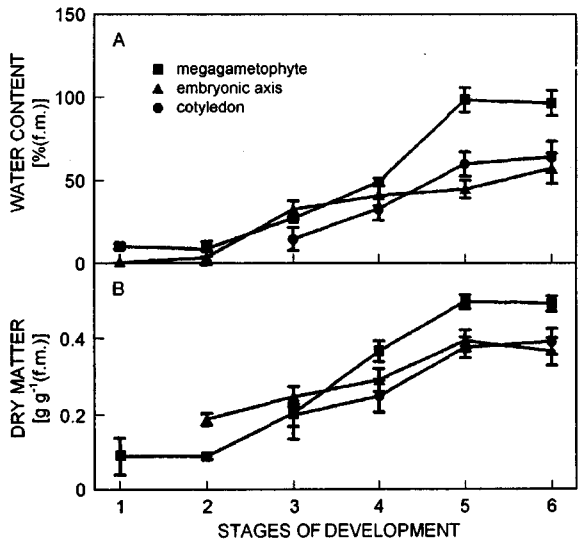

Fig. 2. Water content (*A*) and dry matter (*B*) during seed development of *A. angustifolia*. *Vertical bars* denote standard deviations of the mean; $n = 10$.

during the period of cotyledon formation (stage 3). In the mature seed (stage 6) the water content of the megagametophyte (96.6 %) was significantly larger than in the cotyledon and embryonic axis (64.2 % and 57.4 %, respectively). The gametophyte constitutes approx. 97 % of the mature seed (fresh matter). Thus, water content of the whole seed (fresh matter) corresponds approximately to the water content of the gametophyte. The increase in dry matter accumulation was slight in the embryonic axis throughout embryogenesis (Fig. 2*b*), while the megagametophyte increases in dry matter after cotyledon formation. A period of rapid gain in the megagametophyte dry matter occurred from stage 1 to stage 4, as a result of the synthesis and deposition of storage material (Bewley and Black 1994).

Protein: The storage proteins of *A. angustifolia* started to accumulate in the megagametophyte when embryos were growing into the corrosion cavity of the gametophytic tissue (Fig. 3*a,b*). Soluble protein accumulation was slower in the megagametophyte than in the embryonic axis (Fig. 3*a*). There was a lag phase from stage 2 to stage 3 for both tissues, while the cotyledons developed. Following this phase, the amount of soluble protein in the embryonic axis changed drastically. The embryonic axis showed the highest values of soluble proteins in the mature seed, but these values did not represent increasing dry matter, when compared with the megagametophytes (Fig. 2*b*).

Accumulation of insoluble proteins in the embryonic axis increased rapidly at stage 3 (Fig. 3*b*), when the embryos developed cotyledons. The megagametophyte showed slight changes in insoluble protein contents after cotyledon formation (stage 3). Although crystalloids are the main storage proteins for many conifers (Attree and Fowke 1993), the amount of soluble and insoluble proteins in mature seeds of *A. angustifolia* was similar, suggesting that proteins from the matrix and crystalloids have the same importance as storage reserves.

Megagametophytes of *Pinus monticola* stored 60.5 % of the total insoluble proteins (crystalloid), while the majority of soluble proteins (92.7 %) were present in the cotyledons and embryonic axis tissues (Gifford 1988). In *Pinus contorta* seeds crystalloids accounted for approximately 70 % of insoluble proteins, and the female gametophyte stores 90 - 95 % of this fraction (Lammer and Gifford 1989, Groome *et al.* 1991).

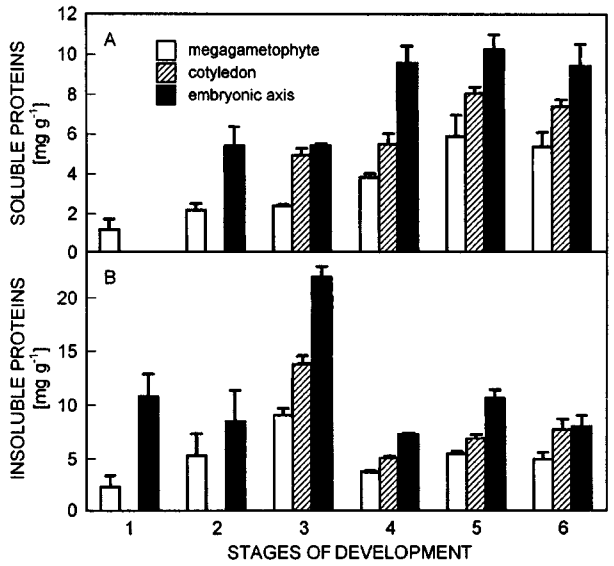

Fig. 3. Soluble (*A*) and insoluble (*B*) proteins during seed development of *A. angustifolia*. *Vertical bars* denote SD of the mean; $n = 3$ (see Materials and methods for detail).

The major storage reserves in the mature seeds of *A. angustifolia* are saccharides (*ca.* 70 % of dry matter) and N-compounds (*ca.* 20 % of dry matter) (Ferreira and Handro 1979, Ferreira *et al.* 1979). Quantitatively, proteins constituted nearly 5 % of the fresh matter of mature *A. angustifolia* seeds (Table 1). Although many conifers are characterized by storing proteins specifically in the megagametophyte (Flinn *et al.* 1991), cotyledons as well as the embryonic axis of *A. angustifolia* showed greater amounts of soluble and insoluble proteins than the megametophyte (Table 1).

Histochemical analysis of protein bodies of the related species *Agathis australis*, showed that crystalloids and globoids looked similar to those in *Pseudotsuga menziesii* and *Picea glauca* (Owens *et al.* 1997). These proteins stored in the seed might be used during development of embryos; this can be suggested from their abundance in the mature embryo and their rapid degradation upon onset of germination (Hakman *et al.* 1990).

Amino acids: The pool of free amino acids of *A. angustifolia* changed during tissue morphological changes along the development (Fig. 3*a*), with the greatest values during the period of cotyledon formation (stage 4). In this period, the insoluble proteins decreased drastically in the embryonic axis (Fig. 3*b*) while soluble proteins increased (Fig. 3*a*). In the mature seed (stage 6), most of the free amino acids are concentrated in the embryo (embryo axis + cotyledons, Fig. 4*a*).

In the pool of free amino acids, glutamic acid, aspartic acid, serine and alanine predominated, with the highest concentrations in stages 4 and 5 (Fig. 4*b*). In the mature seed (stage 6), cotyledons and embryo axis showed higher amounts of free amino acids than megagametophyte (Table 2). Some amino acids are represented similarly in all parts of the seed, but others, such as asparagine, phenylalanine and particularly arginine and GABA, showed higher contents in the embryo in relation to the gametophyte. Arginine and GABA are related with polyamine metabolism, arginine being a precursor of polyamines (Galston and Kaur-Sawhney 1995) and GABA can be synthesized from putrescine (Hausman *et al.* 1997) or from glutamic acid (Satya Naraian and Nair 1990). GABA and arginine are the predominant N-forms in developing fruits (Valle *et al.* 1998).

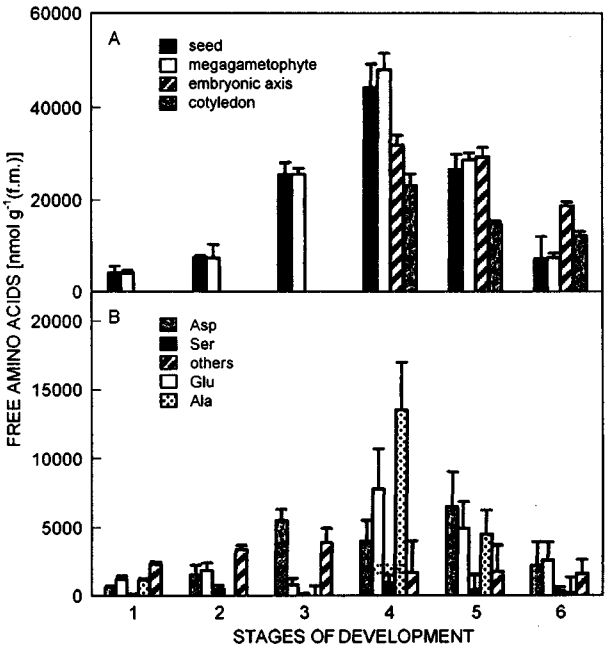

Fig. 4. Total free amino acids in seed tissues (*A*) and changes in the amino acid profile (*B*) during the development of *A. angustifolia* seed. *Vertical bars* denote SD of the mean (see Materials and methods for detail).

Table 1. Soluble and insoluble proteins [mg g^{-1}(f.m.)] in the megagametophyte, embryonic axis and cotyledons of mature seeds and the percentage protein content of each tissue in the protein content of the whole seed in parentheses.

Proteins	Megagametophyte	Embryonic axis	Cotyledons
Soluble	5.3 (96.2 %)	9.4 (1.0 %)	7.3 (2.8 %)
Insoluble	4.8 (96.1 %)	7.8 (0.9 %)	7.5 (3.0 %)

Asparagine has been described as the major transported and stored nitrogen compound in many higher plants due to its solubility and low reactivity (Urquhart and Joy 1981). Seed tissues of *A. angustifolia* did not show great amounts of asparagine (Table 2) perhaps due to its rapid incorporation into proteins for growth (Calanni *et al.* 1999). An increase in aspartic acid following mid-embryogenesis (Fig. 4*b*), suggests that this amino acid might be involved in translocation and would be a precursor for the synthesis of certain amino acids such as asparagine, threonine, isoleucine, lysine and methionine (Heldt 1997). In *Picea engelmannii*, aspartic acid accumulation was associated with water deficiency (Calanni *et al.* 1999). In germinating seeds of *Pinus banksiana*, the megagametophyte represents 60 % of the nitrogen source for seedling growth, wherein the free amino acids consisted mainly of glutamine and asparagine (Durzan and Chalupa 1976a). The existence of sequential changes in the free amino acid pool in the developing zygotic embryo of *A. angustifolia*, fits with the suggestion that a strong internal genetic control of nitrogen metabolism determines the form of growth, final dry mass and volume, in tissue cultures of *Pinus banksiana* (Durzan and Chalupa 1976b).

Morphological descriptions have been used for the critical step of identifying specific responsive tissues of conifers for *in vitro* culture (Roberts *et al.* 1989). Mature seeds of *A. angustifolia* maintain relatively high moisture, which reduces their viability to a few months. The contents of soluble and insoluble proteins were similar in the mature seed, and correspond to 4.2 % of fresh matter. The rise of cotyledons showed to be a very distinct phase in embryogenesis, corresponding to physiological and biochemical changes. During this phase, the pool of free amino acids reached the maximum, suggesting a flux from the megagametophyte and embryonic axis to the cotyledon, in which proteins accumulate.

Potentiating somatic embryogenesis in conifers is generally based on empirical knowledge; thus it is necessary to enlarge the physiological and biochemical basis of zygotic embryogenesis with a view to improve conditions of *in vitro* cultures. The use of biochemical criteria, such as changes in the protein and amino acid profile, could provide information on tissue competence or the specific developmental stage most suitable for *in vitro* culture. In many conifers, the optimum time for initiating embryonic cultures is prior to the development of cotyledon primordia in the dominant zygotic embryo (Lu and Thorpe 1987, Becwar *et al.* 1990, Roberts *et al.* 1990). A better understanding of zygotic embryogenesis in *A. angustifolia* could be relevant, leading to successful somatic embryogenesis, in which the initiation of cultures occurs from very early stages of zygotic embryos (Astarita and Guerra 1998).

Table 2. Contents of free amino acids [nmol g^{-1}(f.m.)] in the mature *A. angustifolia* seed. In the whole seed the megagametophyte represents 97.7 %, cotyledons 1.7 % and embryonic axis 0.6 % of the total seed fresh matter (means ± SE, $n = 3$).

Amino acids	Megagametophyte	Embryonic axis	Cotyledons	Whole seed
Aspartic acid	2347 ± 64	2829 ± 113	2667 ± 107	2355 ± 94
Glutamic acid	2738 ± 82	2802 ± 84	2070 ± 62	2726 ± 82
Asparagine	60 ± 4	205 ± 14	152 ± 11	63 ± 4
Serine	456 ± 25	661 ± 36	794 ± 44	464 ± 26
Glutamine	206 ± 10	583 ± 29	633 ± 31	216 ± 11
Histidine	77 ± 6	79 ± 6	64 ± 5	77 ± 6
Glycine	135 ± 6	244 ± 12	221 ± 11	137 ± 7
Arginine	174 ± 11	1795 ± 109	1576 ± 96	208 ± 13
Threonine	88 ± 3	169 ± 5	162 ± 5	90 ± 3
Alanine	355 ± 29	761 ± 62	900 ± 73	368 ± 30
GABA	59 ± 3	941 ± 27	978 ± 28	80 ± 2
Tyrosine	45 ± 3	200 ± 12	183 ± 11	49 ± 3
Methionine	47 ± 4	102 ± 6	101 ± 6	48 ± 3
Tryptophan	6 ± 1	54 ± 2	56 ± 3	7 ± 1
Valine	173 ± 8	332 ± 15	388 ± 17	178 ± 8
Phenylalanine	79 ± 7	233 ± 19	201 ± 16	83 ± 7
Isoleucine	107 ± 10	167 ± 16	210 ± 19	109 ± 10
Leucine	132 ± 10	202 ± 15	221 ± 17	135 ± 10
Lysine	208 ± 3	756 ± 12	733 ± 12	221 ± 4
Ornithine	96 ± 6	69 ± 4	69 ± 4	96 ± 6
Total	7588	13184	12379	7710

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