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The Effects of Ascorbic Acid on White Spruce Somatic Embryogenesis

by

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Abstract

In the last few years, efforts have been directed towards the utilization of somatic embryogenesis as a tool for the in vitro propagation of white spruce (*Picea glauca*). One of the main obstacles encountered towards this goal is the low percentage of somatic embryos able to convert into viable plantlets. From this thesis, it emerges that ascorbic acid (AA) plays a fundamental role during the post-embryonic growth of white spruce somatic embryos. Studies on AA metabolism clearly indicate that the synthesis of this metabolite increases during the early stages of embryo germination in both zygotic and somatic white spruce embryos. In somatic embryos the increase in AA synthesis is positively correlated to the performace of the embryos in the germination medium. High levels of endogenous AA, in fact, were observed in embryos characterized by a high percentage of conversion. Furthermore, exogenous applications of AA were found to improve the post-embryonic performance of those embryos characterized by a low conversion frequency, by inducing growth in "poorly organized" shoot apical mersitems. The mode of action of AA during these processes was investigated. Experimental manipulations of the endogenous AA levels demonstrated that high levels of AA are required to reduce the accumulation of phenolics at the shoot poles, most likely by regulating the activity of major cellular peroxidases. Low peroxidase activity would result in the relaxation of the cell wall components, thus allowing for cell elongation and cell division of the meristematic cells. Additional studies have also demonstrated that AA induces growth in the apical meristems by regulating purine nucleotide biosynthesis. The improved ability of AA-treated embryos to utilize purine bases and nucleosides for nucleotide biosynthesis indicates that AA increases the activity of the purine salvage pathway. High levels of nucleotides are required for active DNA synthesis occurring in the meristematic regions of AA-treated embryos. The extensive utilization of thymidine for DNA synthesis observed in the presence of high levels of endogenous AA confirms this notion. Overall, the findings emerging from this thesis represent a valuable contribution towards the improvement of white spruce somatic embryogenesis.

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Abbreviations

A	Adenine
AA	Ascorbic acid
AAO	Ascorbic acid oxidase
AAP	Ascorbic acid peroxidase
ABA	Abscisic acid
Abs.	Absorbance
ACC	1-aminocyclopropane-1-carboxylic acid
AEC	3-amino-9-ethyl carbazole
AE medium	von Arnold and Eriksson medium
AFR	Ascorbic acid free radicals
AFRR	Ascorbic acid free radical reductase
AK	Adenosine kinase
AMP	Adenosine monophosphate
APRT	Adenosine phosphoribosyltransferase
AR	Adenosine
ARN	Adenosine nucleosidase
ATP	Adenosine triphosphate
AVG	aminoethoxyvinyl-glycine
BA	N^6-benzyladenine
BAW	Butanol-acetic acid-water
BSA	Bovine serum albumin
C	Control
CTP	Cytidine triphosphate
2,4-D	2,4-Dichlorophenoxyacetic acid
d	Days
DHA	Dehydroascorbic acid
DHAR	Dehydroascorbic acid reductase

DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FAA	Formaldehyde-ethanol-acetic acid
FP	Ferulic acid peroxidase
FW	Fresh weight
germ.	Germination
GDP	Guanosine diphosphate
GL	L-galactono-γ-lactone
GP	Guaiacol peroxidase
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized)
HF	Hormone-free medium
HPLC	High performance liquid chromatography
HRPG	Hydroxyproline-rich glycoproteins
IK	Inosine kinase
IMP	Inosine monophosphate
IR	Inosine
L	Lycorine
LM	Litvay medium
min.	Minutes
MS	Murashige and Skoog medium
N	No shoot or root developed
NAA	α-naphthalene-acetic acid
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NPT	Nucleoside phosphotransferase
OA	Orotic acid
OMP	Orotate monophosphate
OPRT	Orotic acid phosphoribosyltransferase

PAS	Periodic acid Schiff
PCA	Perchloric acid
PCNA	Proliferating cell nuclear antigen
PDT	Partial drying treatment
PEG	Polyethylene glycol
PRAICA	Phosphoribosylaminoimidazole carboxamide
PRPP	5'-Phosphoribosylpyrophosphate
PVP	Polyvinylpyrrolidone
R	Root growth only
R-5-P	Ribose-5'-phosphate
S	Shoot growth only
T	Thymidine
TBO	Toluidine blue O
TDP	Thymidine diphosphate
TK	Thymidine kinase
TMP	Thymidine monophosphate
TTP	Thymidine triphosphate
U	Uracil
UDP	Uridine diphosphate
UK	Uridine kinase
UMP	Uridine monophosphate
UPRT	Uracil phosphoribosyltransferase
UR	Uridine
UTP	Uridine triphosphate

CHAPTER ONE

Introduction

White spruce somatic embryogenesis

White spruce is one of the most widely distributed conifers in North America. Besides representing an ecologically valuable species, white spruce is also extensively utilized by the forestry industry for wood and lumber production (Hosie, 1979). This is why, in the last few years, there has been an increasing interest towards the development of in vitro procedures that would allow for rapid multiplication of this species and a large-scale production of superior materials for reforestation.

Somatic embryogenesis is the process by which haploid or diploid cells are induced to generate embryos in culture. This process leads to the production of bipolar structures which are characterized by a well defined root/shoot axis (see Thorpe and Stasolla, 2001). In contrast to flowering plants, where generation of somatic embryos was reported more than 40 years ago (Reinert, 1958; Steward et al., 1958), somatic embryogenesis in conifers is a relatively recent event. Among coniferous species, white spruce was one of the first to be cultured and propagated in vitro. Durzan (1980) observed the development of embryo-like structures from cultured white spruce cells. Such cell aggregates, however, were not able to undergo further maturation. Generation of mature somatic embryos of white spruce, characterized by well defined shoot and root poles and expanded cotyledons, was reported seven years later by two independent groups (Hakman and Fowke, 1987; Lu and Thorpe, 1987).

The understanding that the post-embryonic performance of somatic embryos was strictly dependent upon their maturation conditions, has led to a tremendous effort towards the optimization of new protocols for the production of embryos with superior quality and improved germination (radicle emergence) and conversion (radicle emergence

and production of new leaf primordia) (see Attree and Fowke. 1993). Furthermore. the design of new rational media and manipulations of the culture conditions responsible for such improvements, can be mainly attributed to comparative studies between zygotic and somatic embryogenesis. Knowledge gained about the unique physiological and biochemical environment associated with zygotic embryo development (see Kong et al., 1999) has been a determinant for improving the overall in vitro embryogenic process of white spruce. As a result, many of the culture conditions, including choice of explant, types and levels of growth regulators. osmolarity, and other major constituents of the maturation medium have been optimized for white spruce and other spruces (reviewed by Thorpe and Harry. 2000; Stasolla et al., 2001a. b). Today. in fact. the production of white spruce somatic embryos is a relatively easy process. This is probably the main reason why white spruce somatic embryogenesis is considered by many the best model system for studying embryo development in conifers. The large number of morphogenic. physiological, and biochemical investigations conducted on this system confirms this notion (reviewed by Stasolla et al., 2001a. b).

Establishment and regeneration of white spruce somatic embryos

Establishment and regeneration of white spruce somatic embryos are successfully achieved through a continuous series of three developmental steps: (i) induction and maintenance of embryogenic tissue. (ii) embryo maturation and (iii) embryo conversion (Fig. 1.1).

Induction and maintenance of embryogenic tissue

Initiation of embryogenic tissue in white spruce, as well as in other coniferous species. is generally achieved from juvenile tissue. In the majority of reports. immature

zygotic embryos are utilized as explants (Hakman and Fowke, 1987; Lu and Thorpe, 1987), although production of embryogenic tissue at high frequency (40%) was later obtained from mature embryos dissected from seeds, which had been stored for 11 years (Tremblay, 1990). Attempts to initiate embryogenic tissues from more mature and differentiated explants of white spruce have been elusive. The only exception is the utilization of excised cotyledons from seedlings (Attree et al. 1990; Lelu and Bornman 1990). Induction of embryogenic tissue in white spruce is commonly achieved on a variety of media, depending on the explant utilized. Usually AE (von Arnold and Eriksson. 1981) and LM (Litvay et al., 1981) media are used for immature and mature embryos respectively, whereas Lelu and Bornman (1990) also reported the utilization of MS (Murashige and Skoog, 1962) medium for excised cotyledons. Sucrose (1-3%), together with 2.4-dichlorophenoxyacetic acid (2.4-D) (10 μM) and N^6-benzyladenine (BA) (5 μM) are also required for the induction process. Inclusion of α-naphthalene-acetic acid (NAA) has also been reported for generating embryogenic tissue from mature tissue (Lelu and Bornman. 1990). Embryogenic tissue. commonly initiated from the hypocotyl of the zygotic embryos utilized as explants. becomes visible after 4-6 weeks of incubation in darkness. The embryogenic mass. translucent in colour. is composed of an aggregate of cells from which early filamentous embryos protrude. and these are similar in morphology to their zygotic counterparts. They are characterized by a small embryogenic head, composed of highly cytoplasmic cells, subtended by an elongated suspensor region of vacuolated cells (Lu and Thorpe, 1987).

Maintenance of embryogenic tissue occurs on a medium very similar to the induction medium, the only difference being the lower concentrations of auxin and cytokinin. and a reduced sucrose concentration (see Thorpe and Harry. 2000). Regular transfers every 10-14 days on solid medium. or 7 days in liquid medium. are required to avoid browning, as also observed for other coniferous species (Gupta and Durzan. 1987). Prolonged subcultures, especially in liquid medium. have been often associated with changes in embryogenic potential of white spruce tissue, resulting in a lower production of embryos (Dunstan et al., 1993). Alternative methods of tissue maintenance. reported

in white spruce, involve cryopreservation (Kartha et al., 1988), long-term storage in gas-impermeable serum-capped flasks at room temperatures (Joy et al., 1991a), and storage at low temperatures, i.e. 0-10° C (see Attree and Fowke, 1993).

Embryo maturation

Removal of auxin and cytokinin, together with applications of abscisic acid (ABA) and increased osmolarity in the medium are required for production of cotyledonary stage embryos in white spruce, as well as other coniferous species (Thorpe and Harry, 2000). In white spruce, reduction of auxin and cytokinin levels is commonly achieved by culturing immature somatic embryos for one week in a medium devoid of plant growth regulators prior to ABA treatment. ABA is also required for the development of white spruce somatic embryos. Responsiveness of the embryogenic tissue to ABA has been found to vary among different genotypes. Usually, high levels of ABA (40-50 µM) are required to promote somatic embryo maturation in white spruce (Joy et al., 1991b; Kong and Yeung, 1992), although a similar effect was achieved with a lower concentration of ABA (12 µM) (Attree et al., 1990).

Besides ABA, high osmolarity in the medium could also be necessary during white spruce somatic embryo maturation, especially in those cases where precocious germination occurs (Gupta and Pullman, 1991). Restriction of water uptake in vitro can be achieved with either permeating osmotica, such as sucrose, mannitol, amino acids, and inorganic salts, or by non-permeating osmotica such as polyethylene glycol (PEG) and dextran (Attree and Fowke, 1993). For white spruce, PEG is the most commonly used osmoticum agent (Attree et al., 1991). Several studies have documented both quantitative and qualitative improvements of white spruce somatic embryos matured on a ABA-containing medium in the presence of PEG (Attree et al., 1991; Kong and Yeung, 1995). Today, the combined application of ABA and PEG is the most commonly used routine for promoting maturation of somatic embryos of several spruce species, including white spruce (Attree et al., 1991; Kong and Yeung, 1995; Find, 1997). Although

beneficial for somatic embryo maturation, the effect of PEG on post-embryonic development is controversial, as low conversion rates were obtained for somatic embryos of white spruce (Kong and Yeung, 1995) and other species of spruce (Find, 1997; Bozhkov and von Arnold, 1998) matured in the presence of PEG.

Desiccation is a natural process occurring at the late stages of seed maturation, and represents an important transition that terminates seed development and initiates the germination process (Kermode, 1990). Morphologically developed white spruce somatic embryos cannot successfully germinate and convert into viable plantlets unless they undergo a drying period. In white spruce, as well as in other coniferous species, somatic embryos cannot tolerate a dramatic loss of moisture content during the drying period. As such, gradual and limited water loss can be achieved through a partial drying treatment (PDT) at high relative humidity (Roberts et al., 1990). During this process, in which the cotyledonary embryos are kept for 10 days in the inner wells of tissue culture plates, while the outer wells are filled with water (Roberts et al., 1990), the moisture content declines by only 20% (Kong, 1994). Partially dried embryos of white spruce had a more synchronous and a higher percentage (80%) of conversion frequency, compared to embryos germinated without prior PDT (2-2.5%) (Kong, 1994).

Embryo conversion

Conversion (radical emergence and formation of new leaf primordia) of white spruce somatic embryos is generally achieved after transferring the partially dried embryos onto a medium devoid of growth regulators. Conversion frequency is strictly dependent upon the genotype of the cell line and the quality of the embryos obtained during the maturation period (Kong and Yeung, 1992). Other factors, including light, temperature, and position on the medium also contribute to the post-embryonic performance of the embryos (Hay and Charest, 1999).

Fig. 1.1. Diagram showing the different steps required for the induction. maturation. and conversion of white spruce somatic embryos. 2.4-D. 2.4-dichlorophenoxyacetic acid: BA. N^6-benzyladenine: ABA. abscisic acid.

Immature and mature zygotic embryos

+2.4-D
+BA

INDUCTION

Embryogenic tissue

MAINTENANCE

±2.4-D
-BA

Embryogenic tissue

7 days — Hormone free medium (-2.4-D: -BA)

7 days — Liquid maturation medium (+ABA)

MATURATION   40 days — Solid maturation medium (+ABA)

Fully developed somatic embryos

10 days — Partial drying treatment (PDT)

CONVERSION

Mature somatic embryos → **Plantlets**

Solid medium (-growth regulators)

Morphogenic events during white spruce somatic embryogenesis

As reported by Nagamani et al. (1987). initiation of white spruce somatic embryo development is characterized by asymmetric cell divisions within the embryogenic tissue. The two daughter cells originating by such divisions are morphologically distinct, as one is small and densely cytoplasmic, whereas the other is larger and vacuolated. As a result of their different morphology, these cells embark on different developmental pathways, as cell divisions of the densely cytoplasmic cell will give rise to the embryo proper, whereas divisions in the vacuolated cell will form the suspensor (Nagmani et al. 1995). Structural studies have revealed that histodifferentiation in the developing white spruce somatic embryos occurs at the early-filamentous stage with the formation of the protoderm, the outermost layer of cells of the embryo proper (Kong et al. 1999). Anticlinal divisions within the protoderm make this layer easily recognizable from the adjacent internal cells, where both anticlinal and periclinal divisions occur. The formation of the protoderm is an important event during somatic embryo development, as it may regulate further embryo growth and development (Yeung, 1995).

Differentiation of both apical meristems and procambium occurs at the filamentous stage of embryo development. Upon further development, a ring of 5-8 cotyledons emerges from the proximal portion of the embryo proper, and this delineates the beginning of the cotyledonary phase of embryo development. During the late stages of embryo development, growth of the suspensor is reduced, and in fully mature embryos, the suspensor region consists of a few cells connecting the embryos to the embryogenic tissue.

Apical meristem differentiation represents an important event during somatic embryogenesis. In white spruce, differentiation of the shoot apical meristem occurs at the filamentous stage of embryo development, when a dome-shaped projection emerges from the proximal portion of the embryo (Kong et al., 1999). Within the differentiating shoot meristem, the apical layer is composed of large, densely cytoplasmic cells, whereas the

subapical layers are formed by vacuolated cells. Upon further development, vacuoles in the subapical layers decrease in size (Kong, 1994). The subapical cells may create a "special environment" for the differentiation of the overlying cells (Kong and Yeung, 1992; Yeung et al.. 1998; Kong et al.. 1999). Differences in shape of the shoot apical meristems are often observed among embryos of different cell lines. as flat- or dome-shaped meristems are observed. In general. the structure of the shoot apical meristems of cotyledonary stage somatic embryos (Kong and Yeung. 1992) appears less organized than those observed in the zygotic counterparts (Yeung et al.. 1998). Poorly developed shoot poles, often disrupted by the presence of intercellular air spaces. are commonly observed in white spruce somatic embryos (Kong and Yeung. 1992). It has also been demonstrated that the structure and organization of the shoot apical meristem of developing embryos affect the post-embryonic growth. Poor germination and conversion frequencies are often observed in those embryos characterized by poorly developed meristems (Kong and Yeung. 1992). Although not as well investigated. the organization of the root apical meristem of white spruce somatic embryos closely resembles that of their zygotic counterparts. the only exception being a reduced number of initials (Kong. 1994; Yeung et al.. 1998).

Physiological and biochemical events during white spruce somatic embryogenesis

Despite the increasing knowledge on the morphogenic aspects of white spruce somatic embryogenesis. to date, there are only few investigations dealing with the physiology and biochemistry of the process. One of the major obstacles encountered is the fact that physiological responses often differ among different genotypes. thus making any attempt of generalization elusive (Stasolla et al.. 2001a.b). As such. instead of providing a large body of information. these studies have resulted in a broad spectrum of often contradictory data. which. however. have represented a useful tool for improvi..g the manipulation of in vitro embryogenesis. As previously mentioned. one of the major differences between in vitro and in vivo embryo development is their respective

embryonic environments. Thus, physiological and biochemical studies have mainly focussed on comparative investigations between in vitro and in vivo embryogenesis.

One of the major factors affecting the in vivo embryogenic process of white spruce is represented by changes in hormone levels. especially ABA (Kong et al.. 1997). In developing white spruce zygotic embryos. endogenous ABA is low during the initial stages of development. it reaches its maximum level at the midpoint of embryo maturation, and then it declines again during the late stages, as the seed dries (Kong et al.. 1997). The requirement for high levels of ABA. necessary to promote storage product deposition and prevent precocious germination of developing embryos. has been reported in several studies (Bewley and Black. 1994). In white spruce. differences in ABA levels have been observed between zygotic and somatic embryonic environments. Comparative studies between developing seeds and somatic embryos of white spruce have revealed higher levels of endogenous ABA in the former. Such a difference was mainly ascribed to the presence of the megagametophytic tissue. which was found to be the major source of ABA for the developing zygotic embryo (Kong et al.. 1997. 1999). It is therefore obvious that the high levels of exogenous ABA. needed to promote somatic embryo development in white spruce. replace the ABA supplied by the megagametophytic tissue during in ovulo embryogenesis.

Physiological studies on ABA metabolism have revealed that although commonly applied as a racemic (±) mixture. only (+)-ABA is able to stimulate the maturation of white spruce somatic embryos (Dunstan et al.. 1992). The responsiveness of the tissue to ABA is a very rapid event. as the first responses can be observed a few hours after the treatment (Dong and Dunstan. 1996: Dong et al.. 1997). However. the actual role played by ABA in stimulating maturation of conifer somatic embryos remains unclear. as multiple developmental responses are observed. One of the early responses of ABA-treated tissue is the reduction of cell proliferation and the initiation of embryo development. Studies on purine and pyrimidine nucleotide biosynthesis have been utilized to follow this transition, as nucleotide availability affects nucleic acid synthesis and ultimately cell division (see Ross, 1981). Tracer experiments conducted during white

spruce somatic embryogenesis have revealed that all the pathways of pyrimidine metabolism, i.e. de novo, salvage, and degradation, operate during the process. Specifically, exogenously supplied orotic acid and uridine were extensively utilized for nucleotide and nucleic acid synthesis, whereas a large fraction of uracil was recovered as degradation products, mainly as CO_2 (Ashihara et al., 2000). For purines, it was also observed that both the salvage and degradation pathways were operative during white spruce somatic embryogenesis (Ashihara et al., 2001). Specifically, adenine and adenosine were extensively salvaged for nucleotide and nucleic acid synthesis, whereas inosine was mainly anabolized to degradation products. The active salvage of both adenine and adenosine sharply decreased upon transfer onto the ABA-maturation medium, and this paralleled a decline in specific activity of the major salvage enzymes of adenine and adenosine: adenine phosphoribosyltransferase (APRT) and adenosine kinase (AK) (Ashihara et al., 2001). Thus, exogenously supplied ABA may regulate the transition between cell proliferation and embryo development by affecting nucleotide biosynthesis.

Changes in nitrogen assimilation and amino acid metabolism, together with alterations in the overall pattern of protein accumulation, were also studied in white spruce cultured cells in response to ABA (Joy et al., 1997). White spruce cultured cells have been found able to take up and incorporate both inorganic nitrogen species (NO_3^- and NH_4^-), although NH_4^- was preferentially taken up and utilized throughout the maturation period (Joy et al., 1997). Reduction of NO_3^- to NH_4^- was also observed through ^{15}N NMR spectroscopic studies. Alterations in amino acid composition were also observed during white spruce somatic embryogenesis. The most notable changes in amino acid profile were the increased concentrations of glutamic acid, glutamine, and arginine and the decrease in alanine level as maturation progressed (Joy, 1993). ^{15}N NMR spectroscopic studies also confirmed this trend, as applications of ABA increased resonances for arginine, glutamine and glutamate, as well as aliphatic amines. The accumulation of these amino acids in maturing embryos was mainly due to the increased activity of the glutamine synthase/glutamate synthase (GS/GOGAT) pathway for

assimilating NH_4^- (Joy et al.. 1997). Such changes in amino acid metabolism also reflected a rise in storage protein (Joy et al.. 1991b). Although the increase of storage protein deposition is a common event in both in vivo and in vitro embryo maturation. differences in matrix and crystalloid polypeptides occurred between developing somatic and zygotic embryos (Joy et al.. 1991b: Misra et al.. 1993). Of particular interest was the observation that some major crystalloid polypeptides were not detected in ABA-matured somatic cotyledonary embryos. which showed an overall crystalloid protein profile similar to that of immature zygotic embryos corresponding to the globular-torpedo stage of development (Misra and Green. 1991; Misra et al.. 1993). This lack of maturity of somatic embryos. also reflected in the structure of the protein bodies (Joy et al.. 1991b). indicates that factors other than ABA are responsible for completion of the maturation process in zygotic counterparts. The contribution of these factors. especially osmoticum. to white spruce somatic embryo development has been extensively investigated in the last few years.

If the embryonic environment of zygotic white spruce embryos is high in ABA compared to the somatic counterpart. the reverse is true for ethylene. a gaseous phytohormone which often accumulates during in vitro culture (Kong et al.. 1999). Several studies have revealed the deleterious effect of ethylene during somatic embryo development. Firstly, non-embryogenic lines of white spruce accumulate more ethylene than embryogenic lines (Kumar et al.. 1989). Secondly. accumulation of ethylene during maturation of white spruce somatic embryos has been found to reduce the number of cotyledonary embryos and to increase morphological abnormality in the shoot pole by inducing intercellular air spaces (Kong and Yeung. 1994). Applications of aminoethoxyvinyl-glycine (AVG). an inhibitor of ethylene biosynthesis. resulted in higher yield of embryos and reduction of embryos with abnormal shoot apical meristems (Kong and Yeung. 1995). A similar beneficial effect on white spruce somatic embryo maturation following experimental inhibition of ethylene biosynthesis was also reported by El Meskaoui et al. (2000). Although the influence on ethylene production in vitro by other hormones. especially ABA. remains controversial (Tan and Thimman. 1989: Riov

et al.. 1990; Biddington et al., 1993), a negative feedback has been observed. as high levels of exogenously supplied ABA decreased the levels of ethylene during the first two weeks of white spruce somatic embryo maturation (Kong, 1994).

Besides hormonal composition, the increasing knowledge on the morphogenic role played by the osmoticum during zygotic embryo development has been critical for improving somatic embryo quality. As documented by Yeung and Brown (1982). the liquid endosperm of flowering plants has more negative osmotic values than those of the embryo. Negative osmotic potential was also observed in somatic and zygotic embryos of different coniferous species, including white spruce (Dumont-BeBoux et al.. 1996). This low osmotic potential is important for a slow development of the embryos. necessary for regulating the pattern of histodifferentiation (Yeung, 1995). The requirement for osmoticum during the embryogenic process has been clearly demonstrated in canola microspore-derived embryos, where inclusions of high levels of polyethylene glycol (PEG). a non-permeating osmoticum agent. dramatically improved the quality of the embryos. which were similar in morphology to their zygotic counterparts (Ilic-Grubor et al.. 1998a. b). Utilization of PEG as an osmoticum agent has been extensively reported also during white spruce somatic embryogenesis. Inclusions of PEG (5-10%) together with ABA resulted in a three-fold increase in the maturation frequency of white spruce somatic embryos and produced somatic embryos with superior appearance to those matured with ABA alone (Attree et al.. 1991). Such embryos had an increased tolerance to drying (Attree et al.. 1995). a nine-fold increase in the amount of storage lipid triacylglycerol with a fatty acid composition resembling that of zygotic embryos (Attree et al.. 1992), and a three-fold higher protein content than somatic embryos matured in the absence of PEG (Misra et al, 1993). This physiological "maturity" observed in PEG-treated white spruce somatic embryos was also reflected by the appearance of some of the major matrix and crystalloid polypeptides which were absent from somatic embryos matured in ABA and low osmoticum. but present in mature seed embryos (Misra and Green. 1991: Misra et al. 1993). The effects of ABA and osmoticum in the regulation of somatic embryo development. especially protein synthesis. appear to be additive. In vitro

translation studies showed that crystalloid protein synthesis is first initiated by ABA alone. but sequentially regulated by PEG at a translational or post-translational level (Misra et al.. 1993).

Morphologically mature somatic embryos cannot successfully germinate and convert into viable plantlets unless they undergo a desiccation period. either through applications of non-plasmolysing osmoticum or by the PDT. Although fundamental for the process. it is surprising that there are very few studies dealing with the physiological and biochemical events occurring during the desiccation period. Changes in hormone level are most likely to occur as the embryos dry. Compared to mature embryos. partially dried white spruce somatic embryos had a lower endogenous level of ABA. as well as a reduced sensitivity to ABA (Kong and Yeung. 1995). Such alterations in ABA metabolism may be beneficial for both germination and conversion processes. The poor post-embryonic growth of white spruce somatic embryos following applications of ABA in the germination medium supports this notion. Similarly to ABA. ethylene production was significantly reduced in partially dried embryos (Kong, 1994).

Besides altering hormone level. the partial drying treatment may be required for increasing the ability of white spruce somatic embryos to generate purine and pyrimidine nucleotides in preparation for the resumption of growth at germination (Stasolla et al.. 2001c).

Problems related to white spruce somatic embryogenesis

Despite the increasing knowledge on structural. physiological and biochemical events occurring during the in vitro embryogenic process of white spruce. there are still limitations for a more extensive utilization of this technique as a large scale propagation. Firstly. both quality and quantity of somatic embryos that can be generated from one cell line are strictly dependent upon the genotype of the explant. In many instances. abnormal development can result in structural aberrations of the embryos, such as the absence of apical regions. deformation of the axis. and fused cotyledons. Secondly. mature somatic

embryos often fail to germinate and convert successfully upon transfer onto the germination medium (Kong and Yeung. 1992). This poor post-embryonic performance has been associated with a failure to establish functional apical meristems during maturation. Structural observations have revealed that those embryos that fail to convert are characterized by abnormal shoot meristems unable to resume cell division at germination (Kong and Yeung. 1992). As such. manipulations of culture conditions aimed at improving both embryo quality at maturation and meristem reactivation at germination will benefit the utilization of somatic embryogenesis in coniferous species for propagation.

Role of ascorbic acid in plant growth and development

Ascorbic acid (vitamin C) has been implicated in many processes in both animal and plant systems. The importance of ascorbic acid (AA) as a dietary requirement goes back to the ancient Roman and Greek empires. where many cases of scurvy (vitamin C deficiency) were observed. especially in large cities as a result of insufficient food and lack of fresh fruits and vegetables in the diet (Sauberlich. 1997). Although AA is found among all plants. often in quite large quantities. its biochemical characteristics in plants is still poorly understood. This is due to various and diversified physiological functions played by AA in plant systems.

Ascorbic acid is a powerful antioxidant. Ascorbate can act as a free radical scavenger by removing reactive oxygen species generated by aerobic metabolism and during exposure to pollutants and pesticides. This is particularly important in photosynthesis. where high concentrations of oxygen-derivatives inhibit the development of chloroplasts (Miyake and Asada, 1992).

Ascorbic acid can act as an enzyme cofactor. Besides being utilized as a substrate by 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (reviewed by Gaspar et al.. 1996). AA has been demonstrated to be a cofactor for several hydroxylases. enzymes involved in the synthesis of hydroxyproline. which are found in several cell wall-

associated hydroxyproline-rich glycoproteins (HRPGs), including extensin and arabinogalactan proteins (De Gara et al.. 1991; De Tullio et al.. 1999).

Ascorbic acid can function as an electron donor in several cellular reactions. Specifically. it has been demonstrated that AA can act as an in vitro electron donor for photosynthetic and mitochondrial electron transport. Miyake and Asada (1992) reported the involvement of the oxidized form of AA, dehydroascorbic acid (DHA). in membrane electron transport.

Finally, ascorbic acid is required to carry out fundamental processes such as cell elongation and cell division. Immunolocalization and quantification studies have revealed high levels of AA in meristematic regions (Liso et al., 1988; Kerk and Feldmann. 1995). Furthermore. an increase in the endogenous AA content has been found to promote cell proliferation in several systems. such as *Allium cepa* roots. where AA induces a progression of meristematic cells from G_1 to S (Liso et al.. 1988). tobacco cultured cells (de Pinto et al.. 1999). *Zea mays* roots (Kerk and Feldman. 1995). and cambial cells of *Lupinus albus* roots (Arrigoni et al.. 1997). Conversely. when the AA content is experimentally lowered by lycorine. an inhibitor of the last enzyme of the de novo AA biosynthesis. cells that are normally competent to divide. arrest in G1 phase of the cell cycle (Liso et al., 1984). An AA-dependent regulatory mechanism of the cell cycle has also been observed in animal systems, where AA increases cell proliferation by inducing a general shortening of the cell cycle and stimulates the entry of cells into S phase (Navas and Gomez-Diaz, 1995). Despite the proposed involvement of AA in the regulation of cell division via hydroxylation of proline residues of specific proteins required for the progression of cell cycle (De Tullio et al.. 1999). to date there is no substantial evidence supporting a putative mechanism of action for AA during cell proliferation.

If the reduced form (AA) of the ascorbate pool seems to be involved in cell division. supporting evidence suggests that the oxidized forms, i.e. ascorbate free radicals (AFR) and dehydroascorbic acid (DHA). are required for cell elongation. An increase in cell expansion, vacuolation. and solute uptake was observed in *Allium cepa* roots treated

with exogenous applications of AFR (Gonzales-Reyes et al.. 1994; 1995). Furthermore. high levels of DHA were observed in *Vicia faba* seeds during the second half of seed maturation. characterized by cell elongation (Arrigoni et al.. 1992). Elevated levels of DHA can induce cell expansion either directly. by reacting with the side chains of lysine and arginine residues in the cell wall, thus preventing cross-linking of structural proteins with hemicelluloses and polygalacturonate (Lin and Varner, 1991), or indirectly. by conversion to oxalate which regulates calcium level in the cell wall by the formation of calcium oxalate crystals (Smirnoff. 1996). A lower concentration of calcium would increase cell wall extensibility by reducing cross-linking between polygalacturonate chains. Finally. de Pinto et al. (1999) proposed that the DHA-induced cell expansion is the result of inhibition of cell proliferation. The authors suggested that. as high levels of DHA are lethal to cell metabolism. cells would divert reducing power from important reactions required for cell cycle progression in an attempt to reduce DHA to AA.

Since AA is an essential metabolite implicated in vital cell functions. it is surprising that the pathway of ascorbate synthesis in plants still remains to be established. So far only a tentative pathway has been proposed. where AA is synthesized from guanosine diphosphate (GDP)-mannose. This pathway shares GDP-sugar intermediates with the synthesis of cell wall polysaccharides and glycoproteins that contain D-mannose. L-galactose. and L-fructose. Synthesis of AA is started in the cytoplasm by sequential reactions that convert GDP-mannose to L-galactono-1.4-lactone (GL) via L-galactose. The final oxidation of GL to AA. catalysed by GL-dehydrogenase. occurs in the inner mitochondrial membrane (reviewed by Smirnoff. 2000) (Fig. 1.2). Production of AA seems to be modulated by feed-back mechanisms, as inhibition of AA synthesis from ^{14}C-glucose was observed after pre-loading pea seedlings with ascorbate (Pallanca and Smirnoff, 2000). Although the mannose pathway predominates in plants, the participation of other precursors cannot be excluded. as demonstrated by the conversion of radio-labelled (methyl)-D-galacturonate and D-glucuronolactone to ascorbate (Loewus. 1999).

Difficulties in the study of the AA biosynthetic pathway arise from the complexity

of AA metabolism. Besides the de novo pathway from sugar molecules. in fact. AA can also be generated through an alternative pathway. which involves the reduction of ascorbate free radical (AFR) and dehydroascorbate (DHA). catalysed by the enzymes ascorbate free radical reductase (AFRR) and dehydroascorbate reductase (DHAR) respectively. Ascorbate free radical reductase (AFRR) uses reduced nicotinamide-adenine dinucleotide phosphate (NADPH) as an electron donor. whereas DHAR activity depends upon the availability of reduced glutathione. Re-conversion of AA to AFR and DHA is mediated by two other redox enzymes; ascorbic acid peroxidase (AAP), an oxygen-peroxide scavenging enzyme. and ascorbic acid oxidase (AAO) (Fig. 1.3).

Objectives of this research

Embryogenesis. the transition between the fertilized egg to the new multicellular generation. is a fundamental process during plant development. Although investigations of the events occurring during embryo maturation and germination are important in increasing our knowledge on how plants grow and develop. there are few studies dealing with these processes. This is mainly due to the fact that besides being small in size. zygotic embryos are embedded in the maternal tissue. thus making any attempt to conduct physiological and biochemical studies difficult. In the last few years. however, somatic embryogenesis has been utilized as an alternative system to conduct studies on the embryogenic process. Besides the large number of embryos obtained in culture. somatic embryogenesis allows experimental manipulations impossible to conduct in an in vivo system. White spruce somatic embryogenesis represents a versatile tool for studying structural and physiological aspects of embryo maturation and germination.

Fig. 1.2. Proposed de novo synthesis of ascorbic acid (AA) via GDP-mannose and L-galactose. Enzymes involved in this pathway are: (A) Glucose phosphate isomerase: (B) phosphomannose isomerase: (C) phosphomannose mutase: (D) GDP-mannose pyrophosphorylase: (E) GDP-mannose-3.5-epimerase: (F) uncharacterised enzymes: (G) L-galactose dehydrogenase: (H) L-galactono-1.4-lactone dehydrogenase. Lycorine inhibits the activity of L-galactono-1.4-lactone dehydrogenase (Modified from Smirnoff. 2000)

Glucose-6-P

A

Fructose-6-P

B

Mannose-6-P

C

Mannose-1-P

D

GDP-mannose ──────────▶

E

Cell wall polysaccharides and glycoproteins

GDP-L-galactose ·············▶

F

L-Galactose

Lycorine

G

L-Galactono-1,4-lactone (GL)   Ascorbate (AA)

H

Mitochondrion
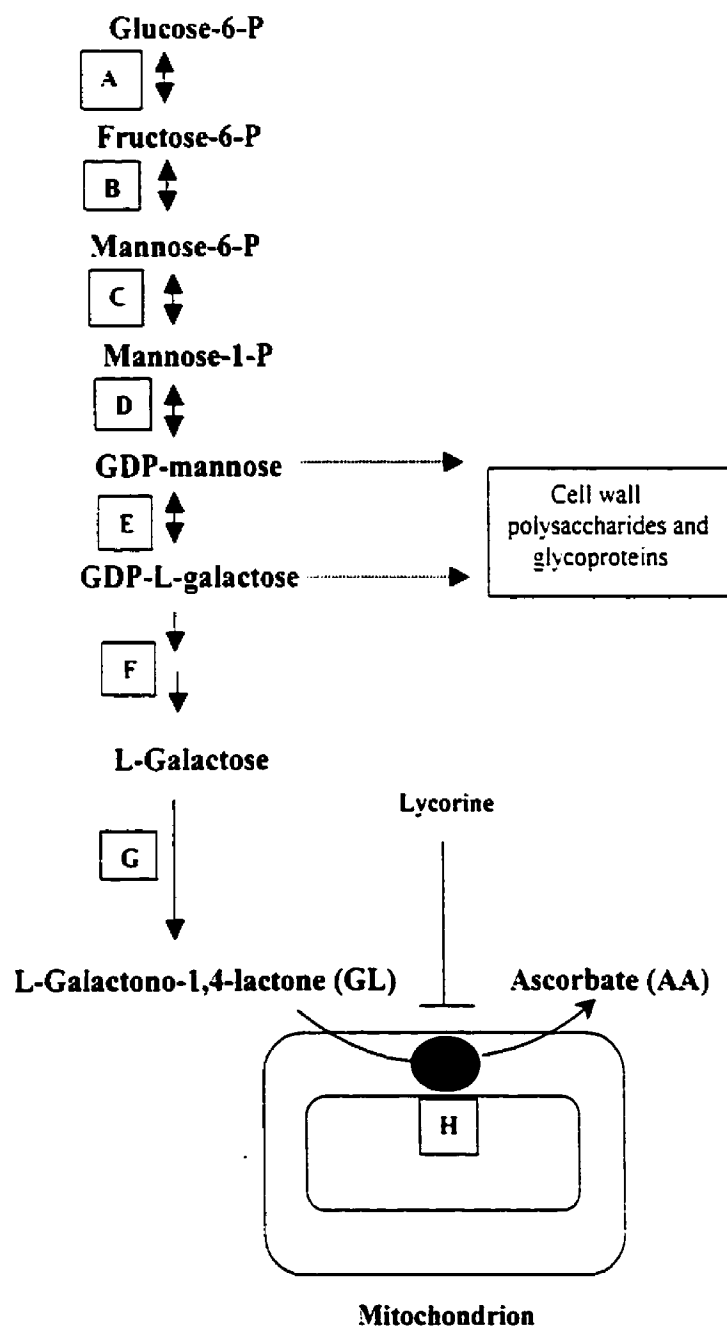
Fig. 1.3. Schematic representation of the ascorbate system in plant cells.
Ascorbate (AA) produced de novo can be oxidized to ascorbate free radicals
(AFR) and ultimately to dehydroascorbate (DHA) by the enzymes ascorbic acid
oxidase (AAO) and ascorbic acid peroxidase (AAP). Reduction of AFR and
DHA to AA is catalyzed by ascorbate free radical reductase (AFRR) and
dehydroascorbate reductase (DHAR) respectively. GL. L-galactono-γ-lactone.

Glucose

de novo biosynthesis

GL

AA

NADP⁻

O₂

H₂O₂

AFRR

AAO

AAP

GSSG

NADPH – H⁻

H₂O

DHAR

AFR

2 GSH

non-enzymatic reaction

DHA

The screening of cell lines with different embryo-forming capacity allows the identification of developmental blocks during the maturation and germination processes. One of such blocks is represented by the failure of somatic embryos to convert into viable plantlets at germination. The post-embryonic performance of mature somatic embryos is strictly genotype-dependent, as conversion frequency varies from line to line and is dependent upon the quality of the apical meristems. Those embryos which fail to convert are in fact characterized by poorly developed apical poles, which are not able to resume cell division at germination (Kong and Yeung, 1992). As AA plays a key role during cell division and proliferation, the main objective of this research is to determine whether experimental alterations of AA metabolism in white spruce somatic embryos result in improved performance at germination. As a first step, studies on AA metabolism were conducted during maturation and germination of white spruce zygotic and somatic embryos. Such comparative studies were necessary to determine whether changes in AA utilization correlate with important developmental phases of the embryogenic process. Further experimental manipulations of AA metabolism, especially during germination were also performed. Particular attention was addressed towards the participation of AA during the reactivation of the apical meristems at germination. Exogenous applications of AA or lycorine, an inhibitor of AA biosynthesis, were used to determine the involvement of AA on cell division of meristematic cells. The effects of such manipulations were assessed by using structural observations and conversion frequencies of the treated embryos. Finally, the mode of action of AA on meristem reactivation was studied by investigating how alterations of AA metabolism affect peroxidase activity and nucleotide and nucleic acid metabolism.

In summary, the main objectives of this research were:

1. To study AA metabolism during zygotic and somatic embryogenesis (Chapter 2)

2. To determine whether exogenous applications of AA induce resumption of mitotic activity in meristem cells of germination somatic embryos (Chapter 3)

3. To determine the mode of action of AA on the reactivation of the apical meristems by investigating:

    A. Changes in peroxidase activity (Chapter 4)

    B. Changes in nucleotide and nucleic acid metabolism (Chapters 5 and 6).

This work was performed with two different lines of white spruce. as the original line (E)WS1. utilized for Chapters 2. 3. and 5. lost its embryogenic potential after two years in culture. For Chapters 4 and 6 the line (E)WSC was employed.

CHAPTER TWO

Ascorbic acid metabolism during somatic and zygotic embryo maturation and germination

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Introduction

Ascorbic acid (AA) is a metabolite required for critical processes in plant growth and development: as such. there are many studies investigating AA metabolism during seed maturation and germination (e.g.. Tommasi and De Gara. 1990: Arrigoni et al.. 1992: De Gara et al.. 1997: Pallanca and Smirnoff. 2000). From these studies. it emerges that alteration of the endogenous AA content and changes of the specific activity of the AA-redox enzymes often correlate with different stages of embryo development. During the initial phases of *Vicia faba* seed maturation. characterized by intense cell division. a large proportion of the total ascorbate pool (AA+DHA+AFR) was found in the reduced form (AA). Upon subsequent development. characterized by cell elongation. most of the total ascorbate pool was composed of dehydroascorbate (DHA) and ascorbate free radicals (AFR). the oxidized forms (Arrigoni et al.. 1992). Therefore. the ability of the developing embryos to metabolically alter the AA/DHA+AFR ratio appears to be important for the embryonic growth (Arrigoni et al.. 1992). Changes in AA metabolism were also observed during the late stages of seed maturation and germination. Fully dried *Vicia faba* seeds (Arrigoni et al.. 1992). as well as dry caryopses of wheat (De Gara et al.. 1997). are devoid of AA. but contain a low amount of ascorbate free radical (AFR) and dehydroascorbate (DHA). together with the respective reducing enzymes: ascorbate free radical reductase (AFRR). and dehydroascorbate reductase (DHAR) (see Fig. 1.3. Chapter 1). The presence of these two AA-recycling enzymes represents an important strategy for providing the germinating embryos with AA. before the reactivation of the de novo AA-biosynthetic machinery (De Gara et al.. 1997). In germinating embryos. in fact.

a large availability of AA is required to sustain both cell division (Liso et al.. 1984: 1988: Citterio et al.. 1994) and cell elongation (Cordoba-Pedregosa et al.. 1996: Arrigoni et al.. 1997). Despite the increasing amount of evidence indicating the active role played by AA during embryo development and germination. at present there is no information dealing with changes of AA metabolism during zygotic and somatic embryogenesis in coniferous plants.

This chapter will investigate changes in AA metabolism occurring during white spruce zygotic and somatic embryo development and germination. The main objectives of this research are to determine whether (1) differences in AA metabolism occur during the in vivo and in vitro embryogenetic processes and (2) a better ability to synthesize and metabolize AA correlates with an increased embryogenic potential of cultured cells and improved germination of somatic embryos. In order to achieve this objective. zygotic embryos and three different white spruce cell lines: a non-embryogenic (NE)WS line unable to produce mature embryos. and two embryogenic lines with low (E)WS1 and high (E)WS2 percentage of embryo germination. were utilized.

Materials and Methods

Plant material

Seed collection and germination

Developing white spruce seeds were collected from the campus at the University of Calgary on a weekly basis from June 16 to August 30. 1999. Seeds. dissected from the cones. were frozen in liquid nitrogen and stored at -80° C. before being processed for AA and DHA+AFR measurements and enzyme assays.

For germination. white spruce seeds (lot # 7431580.1; germinability 92%) were obtained from the National Tree Seed Center (Fredericton. N.B.. Canada). Seeds were soaked in water at 4°C for 24 hours, sterilized in 25% Javex® bleach for 20 min. rinsed three times in sterile water. and germinated on moistened filter papers in the dark at 22°

C. Germinated seeds were collected at days 3, 6, and 12, and the embryos were dissected out. Both embryos and megagametophytes enclosed in the seed coat were processed for AA determination and enzyme assays. As the seed coat was mainly composed of dead tissue, and represented a small fraction of the seed, it was considered as part of the megagametophyte.

Establishment of white spruce cell cultures

White spruce (*Picea glauca* [Moench] Voss) embryogenic and non-embryogenic tissues were generated from mature embryos as described by Lu and Thorpe (1987). Seeds, collected from the campus at the University of Calgary, were surface sterilized in 20% commercial bleach for 20 min and rinsed three times with sterile water. The embryos were then dissected and plated on the (AE) induction medium (von Arnold and Eriksson 1981) (Table 2.1) supplemented with 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 5 μM N^6-benzyladenine (BA), 5% sucrose, and solidified with 0.8% agar, pH 5.8. The stock cultures were maintained in the dark at 26° C for 4-5 weeks. The tissues were then transferred to a liquid maintenance medium (AE medium containing 10 μM 2,4-D, 2 μM BA, and 3% sucrose) and were subcultured every seven days. For these experiments, three distinct cell lines were selected: a non-embryogenic cell line (NE)WS, unable to produce mature embryos, and two embryogenic lines characterized by a low (E)WS1 and high (E)WS2 percentage of embryo conversion frequency.

Promotion of somatic embryo development

Promotion of somatic embryo maturation was achieved by two subsequent transfers of 1 g of tissue into 50 ml of hormone-free (HF) medium for 7 days (AE medium devoid of hormones) and then into 50 ml of maturation medium (AE medium supplemented with 50 μM of abscisic acid [ABA] and 5% sucrose) for 7 days. About 50 mg fresh weight of embryogenic tissue was spread on a sterile filter paper (Whatman no. 1, Maidstone, UK)

Table 2.1. Composition of von Arnold and Eriksson (AE) (1981) nutrient medium utilized for embryogenic tissue induction and maturation and germination of white spruce somatic embryos.

Compound	mg/L
NH_4NO_3	1200
KNO_3	1900
$CaCl_2$-$2H_2O$	180
$MgSO_4$-$7H_2O$	370
$MnSO_4$-$4H_2O$	2.2
$CuSO_4$-$5H_2O$	0.0025
$ZnSO_4$-$7H_2O$	4.05
Na_2-EDTA	12.85
$FeSO_4$-$7H_2O$	5.57
H_3BO_3	0.63
KH_2PO_4	340
KI	0.75
Na_2MoO_4-$2H_2O$	0.025
$CoCl_2$-$6H_2O$	0.0025
Thiamine HCl	5
Nicotinic acid	2
Pyridoxine HCl	1
myo-Inisitol	500
Glutamine	500
Caseine hydrolysate	500
Sucrose	(see Materials and Methods)

and placed on a solid ABA-containing medium (maturation medium solidified with 0.8% agar. pH 5.8). After 40 days of incubation in the dark at room temperature (22° C), mature somatic embryos were characterized by well developed cotyledons (Joy et al., 1991b).

Partial drying treatment

The partial drying treatment (PDT), an essential step for somatic embryo germination (Fig. 1.1, Chapter 1), was carried out as described by Roberts et al. (1990). Mature embryos supported by a small sterile filter paper disk (Whatman no. 1) were placed in the central wells of a 24-well tissue culture plate (Falcon 3847, Frankling Lakes, NJ, USA). The outer wells were filled with sterile water. The plates were sealed with parafilm "M" (American National Can. Chicago. IL. USA) and incubated in the dark at room temperature (22° C) for 10 days.

Germination

Following the PDT, the embryos were transferred onto a germination medium (half-strength AE medium, supplemented with 1% sucrose and solidified with 0.8% agar. pH 5.8). Approximately 25 embryos were placed in each petri dish. Germination was carried out under light conditions (photon flux density of 90-95 μmol m^{-2}s^{-1}: 380-800 nm) with a 16-hour photoperiod. Germinating embryos were collected at regular intervals for the first 10 days and processed for AA-DHA determination and enzyme assays.

Light microscopy

Samples were fixed in 2.5% glutaraldehyde and 1.6% *para*formaldehyde buffered with 0.05 M phosphate buffer. pH 6.9, dehydrated with methyl cellosolve, followed by two changes of absolute ethanol, and then infiltrated and embedded in Historesin (Leica,

Markham. ON, Canada) (Yeung and Law, 1987). Sectioning was carried out with glass knives on a Reichert-Jung 2040 Autocut microtome. Serial longitudinal sections were cut at a thickness of 3 μm. For general histological examinations, the sections were stained with the periodic acid-Schiff (PAS) procedure and counterstained with 0.05% (w/v) toluidine blue O in benzoate buffer, pH 4.4 (Yeung, 1984a). The preparations were examined and photographed with a Leitz Aristoplan light microscope.

AA and DHA+AFR measurements

AA and AFR+DHA were measured according to Zhang and Kirkham (1996). Tissue was ground in cold 5% metaphosphoric acid (1:5 w/v) to prevent AA oxidation and DHA degradation. The homogenate was then centrifuged for 20 min at 16,000 g and the supernatant was collected for the measurements. Total ascorbate (AA+AFR+DHA) was determined after reduction of DHA and AFR to AA with dithiothreitol (DTT), and the concentration of AFR+DHA was estimated from the difference between total ascorbate pool and AA. The reaction mixture for total ascorbate pool contained a 0.1 ml aliquot of the supernatant. 0.25 ml of 100 mM phosphate buffer (pH 7.4) containing 5 mM EDTA and 0.05 ml of 10 mM DTT. After incubation for 10 min at room temperature (22° C), 0.05 ml of 0.5% N-ethylmaleimide was added to remove excess DTT. AA was determined in a similar reaction mixture except that 0.1 ml of H_2O was added rather than DTT and N-ethylmaleimide. Color was developed in both reaction mixtures after addition of the following reagents: 0.2 ml of 10% trichloroacetic acid, 0.2 ml of 44% ortho-phosphoric acid, 0.2 ml of 4% α,α'-dipyridyl in 70% ethanol, and 0.3% (w/v) $FeCl_3$. After vortexing, the mixture was incubated at 40° C for 40 min and the absorbance (A_{525}) of the supernatant was measured spectrophotometrically. A standard curve was developed based on AA in the range of 0-50 μg/ml.

AA biosynthesis

This was measured by incubating the tissue in the presence of 5 mM L-galactono-γ-lactone (GL) (the last precursor of AA [Fig. 1.2 . Chapter 1]). After a 9-hour incubation. the tissue was homogenized in 5% metaphosphoric acid and the ascorbate level was measured. The ability to synthesize AA was calculated by subtracting the AA level of control (-GL) tissue from that measured in the presence of GL.

Enzyme assays

The tissue was homogenized at $4°$ C with a medium containing 50 mM Tris-HCl. pH 7.2. 0.3 M mannitol. 1 mM EDTA. 0.1% bovine serum albumine (BSA). 0.05% cysteine. and 2% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at $4°$ C for 20 min at 16000 g. and the supernatant was collected and utilized for the assays of enzymatic activities as reported by Arrigoni et al. (1992).

Ascorbic acid peroxidase (AAP) (EC 1.11.1.11) activity was measured by following the hydrogen peroxide-dependent oxidation of AA by means of the decrease in absorbance at 265 nm. The reaction mixture contained 50 μM AA. 0.01% H_2O_2. and 50 mM potassium phosphate buffer. pH 6.5.

Ascorbic acid free radical reductase (AFRR) (EC 1.6.5.4) activity was determined by monitoring the rate of NADH oxidation at 340 nm. The reaction mixture contained 0.2 mM NADH. 1 mM AA. and 0.1 M Tris-HCl. pH 7.2. The reaction was initiated with the addition of 0.5 units of commercial ascorbate oxidase (EC 1.10.3.3. Sigma). necessary to convert the endogenous AA to AFR.

Ascorbic acid oxidase (AAO) (EC 1.10.3.3) activity was estimated by the oxidation rate of ascorbate at 265 nm in a reaction mixture containing 50 mM potassium phosphate buffer (pH. 7.0). 0.5 mM EDTA. and 0.15 mM AA in a volume of 1 ml.

Dehydroascorbate reductase (DHAR) (EC 1.8.5.1) activity was estimated by measuring the (GSH)-dependent production of ascorbate at 265 nm. The reaction

mixture was composed of 1 mM DHA, 2 mM GSH, and 100 mM potassium phosphate buffer, pH 6.3.

For all enzyme assays, corrections were made by taking into account changes in absorbance due to non-enzymatic reactions.

Protein contents were measured according to Lowry (1951) using a Bio-Rad protein determination kit (Sigma) and BSA as a standard. The Lowry method was preferred to Bradford because reagents incompatible with the Bradford method were used for protein extraction in Chapter 4.

The data for both AA and DHA quantitation and the specific activity of the ascorbate enzymes represent the mean ± SE of three or more experiments. For statistical analyses, the Student-Newman-Keuls test (Zar, 1974) was utilized.

Results

Somatic embryo maturation and germination

Morphological characteristics of the three cell lines

Histological examinations revealed morphological differences among the three cell lines during the maturation period.

In the non-embryogenic line (NE)WS, small groups of cytoplasmic cells subtended by enlarged, vacuolated cells were observed in the maintenance medium (Fig. 2.1 A). Seven days after transfer into the liquid maturation medium (ABA liq. [day7]) no remarkable structural changes were observed in this line. Starch deposition increased, especially within the vacuolated cells (Fig. 2.1 B). The cytoplasmic cells were unable to undergo further development, and at the end of the maturation period (ABA sol. [day 35]) no mature somatic embryos were produced (Fig. 2.1 C).

In the embryogenic (E)WS1 cell culture, well developed early-club-shaped embryos were already discernible in the maintenance medium. They were characterized by an embryo proper, formed by small cytoplasmic cells, and by a suspensor region

composed of large, vacuolated cells (Fig. 2.1 D). After a few days in the maturation medium (ABA liq. [day 7]) these embryos increased in size. A fully differentiated protoderm was observed in the embryo proper, and a pronounced root cap, composed of vertical tiers of cells, appeared in the proximal region of the embryo. Heavy accumulations of starch were discernible in the root cap (Fig. 2.1 E). At the end of the maturation period, morphologically mature somatic embryos characterized by developed cotyledons were obtained (Fig. 2.1 F). Histological observations of these mature embryos revealed the presence of fully differentiated apical meristems. The shoot apical meristem was flat in shape and it was characterized by an apical layer composed of large cytoplasmic cells. Some intercellular air spaces were observed in the sub-apical portion of the shoot meristem (Fig. 2.1 G). The root apical meristem was composed of large isodiametrical cells which stained less than the surrounding cells. A pronounced starch deposition was observed throughout the root cap and cortex (Fig. 2.1 H). Following a period of partial drying treatment, only 33.5% of these embryos were able to undergo successful germination, i.e. emergence of root and new leaf primordia.

In the other embryogenic line, (E)WS2, small clusters of cytoplasmic cells subtended by a few elongated vacuolated cells were discernible throughout the maintenance period (Fig. 2.1 I). Upon transfer onto the maturation medium (ABA liq. [day 7]), these clusters of cells continued to grow and embryogenic heads started to emerge. Unlike those observed in the (E)WS1 line at this stage of development, these embryo-like structures were poorly organized (Fig. 2.1 J). Embryo development occurred mainly on the solid maturation medium. At the end of the maturation period (ABA sol. [day 35]), in fact, many mature somatic embryos were obtained. These embryos displayed similar morphology to that described for those of the (E)WS1 line. Large cytoplasmic cells formed the apical layer of the shoot apical meristem, and a group of isodiametrical cells delineated the root meristem. The quality of these embryos was better than that of the (E)WS1 embryos, since after the PDT their germination frequency was 78%.

Fig. 2.1. Morphological characteristics of the three white spruce cell lines utilized in this study. Small groups of cytoplasmic cells (arrowhead) subtended by large vacuolated cells were observed in the (NE)WS line in the maintenance medium (**A**). No significant changes in morphology were discernible after 7 days in ABA liquid (**B**), and at the end of the maturation period no mature somatic embryos were observed in this line (**C**). In the embryogenic (E)WS1 line small globular-shaped embryos were present in the maintenance medium (**D**). These embryos increased in size during the first week of maturation. They were characterized by a well differentiated protoderm (arrow) and a large root cap (arrowhead) (**E**). At the end of the maturation period mature somatic embryos characterized by fully developed cotyledons were observed in this line (**F**). Histological examinations of these embryos revealed the presence of fully differentiated meristems. The shoot apical meristem was composed of an apical layer of large cytoplasmic cells (arrow) and by a subapical region often disrupted by the presence of intercellular air spaces (arrowhead) (**G**). Large isodiametrical cells which stained less than the surrounding cells formed the root apical meristem (*) (**H**). In the other embryogenic line (E)WS2, small clusters of cytoplasmic cells were discernible in the maintenance medium (**I**). After 7 days in ABA liquid, embryogenic heads start emerging from these clusters of cells (arrow) (**J**). At the end of the maturation period somatic embryos, similar in morphology to those generated by the (E)WS1 cell culture, were observed in this line. Bar = 50 μm. A. B. D. E: 100 μm. G-J: 2 mm C and F.
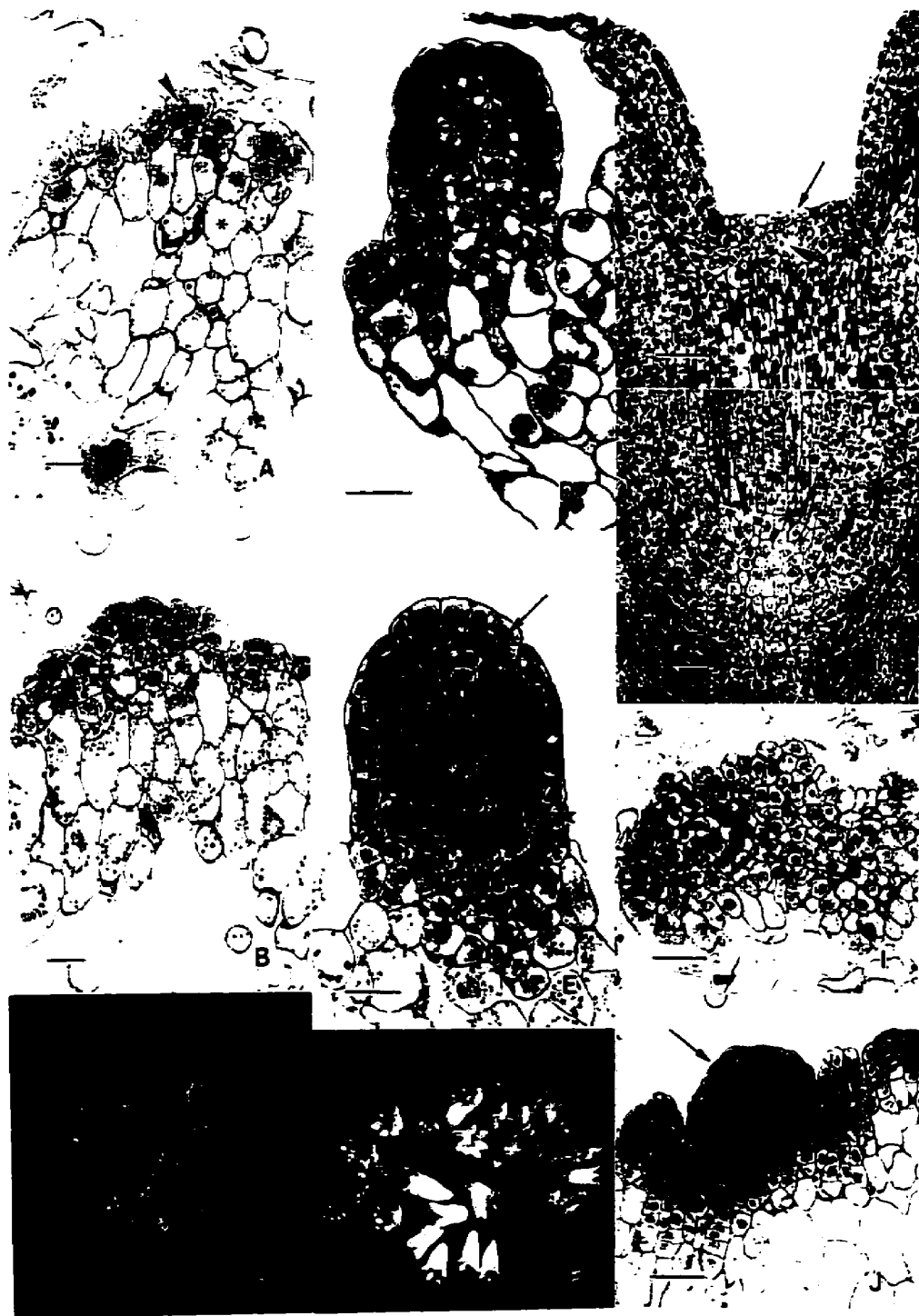
AA metabolism in the maintenance medium

Only small differences in the ascorbate metabolism were observed in the three cell lines in the maintenance (2,4-D) medium. As revealed by statistical analyses, the levels of AA observed in the embryogenic lines (E)WS1 and (E)WS2 were significantly higher than the level observed in the (NE)WS line at 2,4-D (day 4), but not at 2,4-D (day 7) (Fig. 2.2). The total ascorbate pool (AA+DHA+AFR) was within the range of 600-800 nmol g^{-1} fresh weight (Fig. 2.2), and a limited ability to produce AA from its last precursor (GL) was observed in the three cultures (Fig. 2.3 A).

AA metabolism during maturation

The first discernible differences in AA metabolism were observed upon transfer into the ABA-containing liquid medium. The capability of de novo synthesizing AA from its last precursor, L-galactono-γ-lactone (GL), sharply increased in the two embryogenic lines after seven days into the maturation medium, whereas it remained constant in the non-embryogenic culture (Fig. 2.3 A). In the (E)WS1 line, this increment was concomitant with a rise in the endogenous AA level which reached values close to 1500 nmol g^{-1} fresh weight (Fig. 2.2). Within the total ascorbate pool, a shift toward the reduced form, i.e. AA, was also observed in the two embryogenic lines, as revealed by the high AA / DHA ratio. As shown in Figure 2.3 B, throughout the culture period into the maintenance (2,4-D) and hormone-free (HF) media the AA / DHA+AFR ratio was close to 1 in all the three cell cultures. Upon transfer onto the maturation (ABA) medium this ratio increased in the two embryogenic cell cultures, whereas it remained low in the non-embryogenic line. After 35 days on the solid maturation medium this ratio was about 7 and 4.5 for the (E)WS2 and (E)WS1 lines respectively, whereas it was less than 2 for the (NE)WS cell culture (Fig. 2.3 B).

From the data on the activity of the enzymes of the ascorbate system (Table 2.2), it emerges that ascorbic acid peroxidase (AAP), a key enzyme of the AA metabolism

Fig. 2.2. Changes in ascorbate (open bars) and dehydroascorbate (close bars) content during the maturation period of the two embryogenic lines (E)WS1 and (E)WS2 and non-embryogenic line (NE)WS. Values are the means ± SE of three or more experiments. 2,4-D. maintenance medium: HF. hormone-free medium: ABA liq.. liquid maturation medium: ABA sol.. solid maturation medium. Numbers in parentheses indicate days in culture.
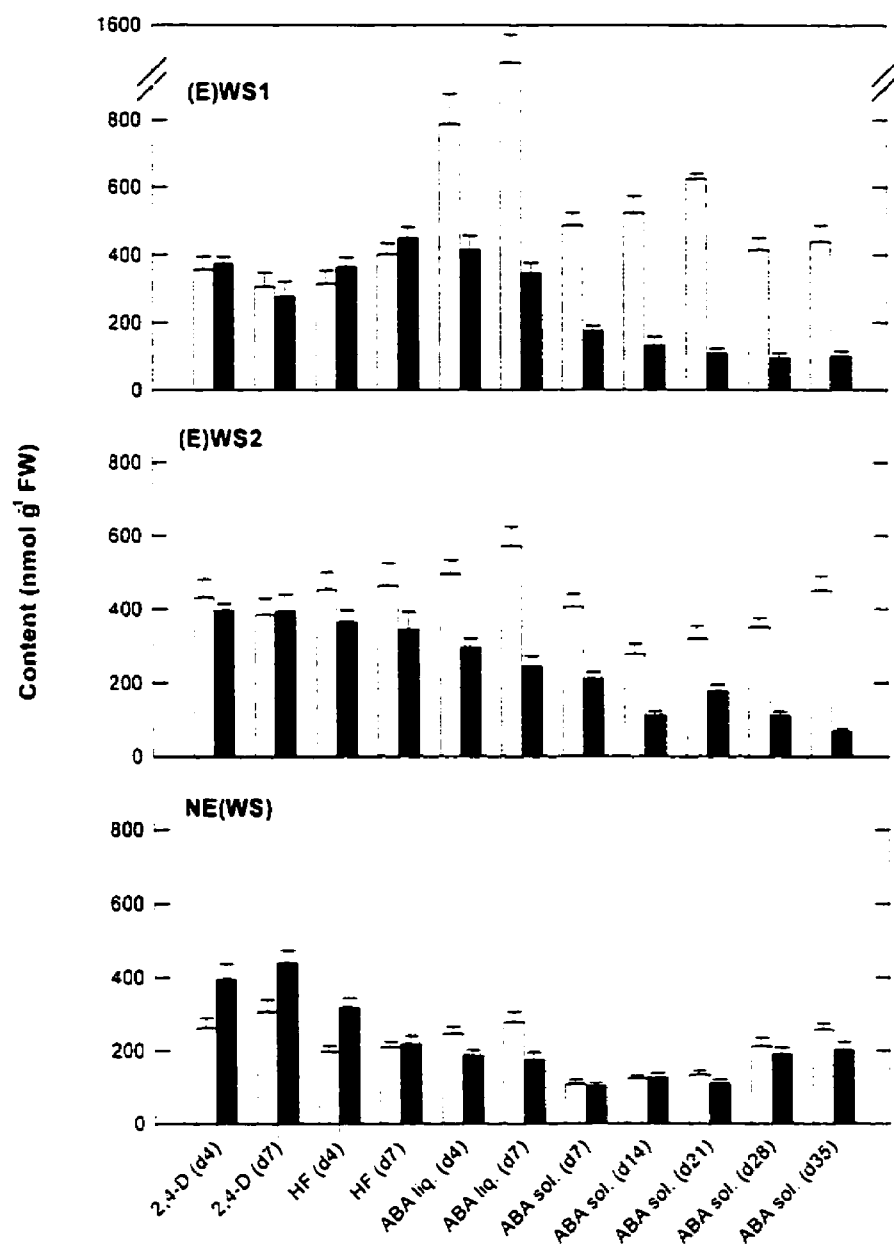
Fig. 2.3. (A) Ability of the two embryogenic lines (E)WS1 and (E)WS2 and the non-embryogenic line (NE)WS to generate ascorbate (AA) from its last precursor L-galactono-γ-lactone (GL) during the maturation period. Values are the means $\pm$ SE of three or more experiments. 2.4-D. maintenance medium: HF. hormone-free medium; ABA liq.. liquid maturation medium: ABA sol.. solid maturation medium. Numbers in parentheses indicate days in culture.

(B) Ratio of the reduced (AA) and oxidized (DHA$\div$AFR) forms within the total ascorbate pool during the maturation period of the two embryogenic lines (E)WS1 and (E)WS2 and the non-embryogenic line (NE)WS. 2.4-D. maintenance medium: HF. hormone-free medium; ABA liq.. liquid maturation medium: ABA sol.. solid maturation medium. Numbers in parentheses indicate days in culture.
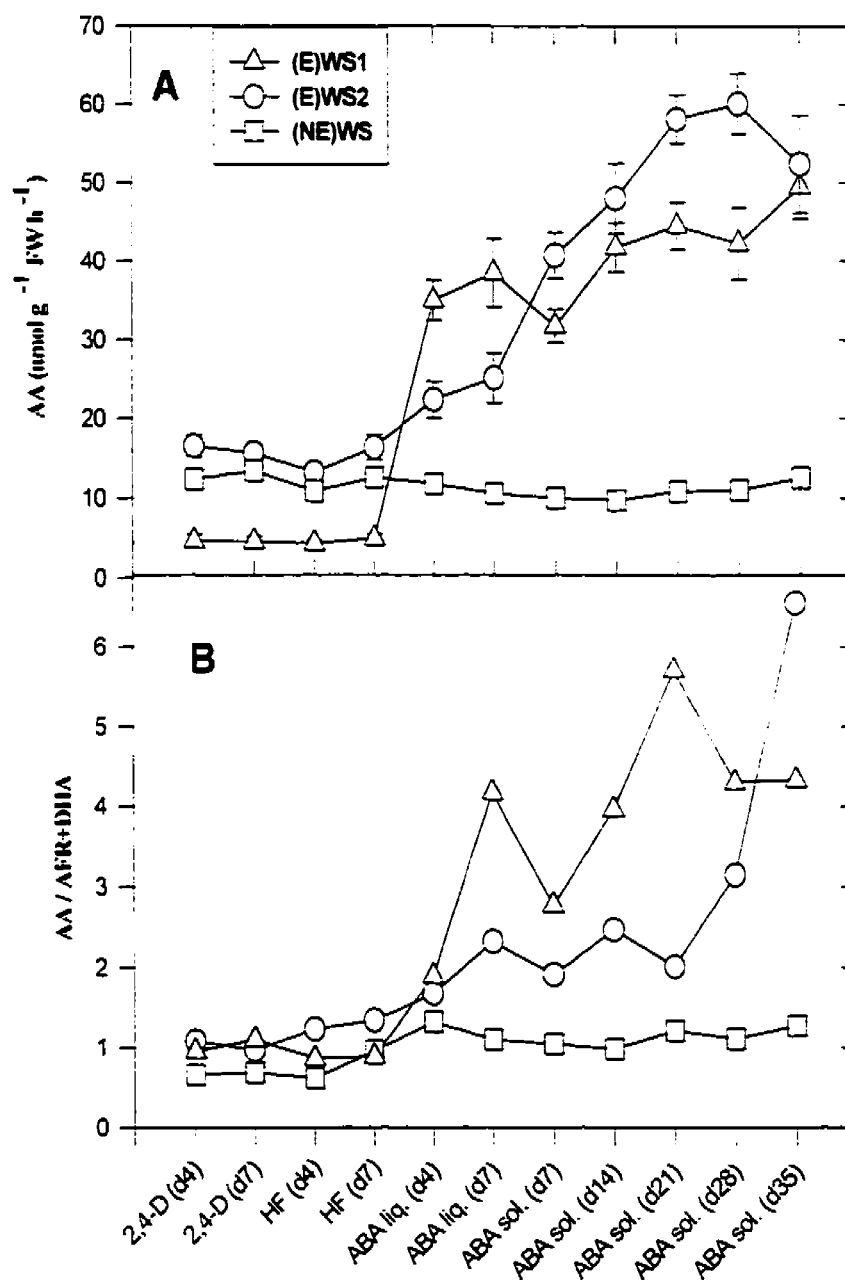
involved in the removal of hydrogen peroxide (Fig. 1.3. Chapter 1), was present at high levels during the early phases of maturation, but it gradually declined in all the cultures upon transfer onto the ABA-containing medium. A similar pattern of activity was found for AFRR, an ascorbate-recycling enzyme that catalyzes the reduction of ascorbate free radicals (AFR) into AA (Fig. 1.3. Chapter 1). It is worth noting, however, that in the two embryogenic lines the decline of AFRR was less dramatic than that of AAP, so that after 35 days on the solid maturation medium its activity was higher than that of AAP. DHAR was not detectable throughout the maturation period (data not shown), whereas AAO activity was only detectable during the late stages of embryo maturation in the (E)WS1 line.

AA metabolism during the partial drying treatment and germination

The total ascorbate pool (AA+DHA+AFR) in the mature embryos of the (E)WS1 and (E)WS2 lines was less than 2 nmol embryo^{-1} (Fig. 2.4). Within this pool, the reduced form (AA) was predominant, as shown by the AA / DHA+AFR ratio (Fig. 2.5 B).

Several changes in the AA metabolism occurred during the partial drying treatment (PDT). Firstly, the total AA pool significantly declined in the embryos of the two lines after 10 days of PDT (Fig. 2.4). This occurred despite a small, but statistically significant increment in the ability to generate AA de novo in the partially dried embryos (Fig. 2.5 A). Secondly, within the total AA pool the AA / DHA+AFR ratio shifted towards the oxidative state (DHA+AFR), reaching values close to 1 at the end of the PDT (Fig. 2.5 B). From the data of the enzyme assays, it emerges that during the PDT the specific activities of both AAP and AFRR decreased, even though at the end of the drying period a large amount of AFRR was still present in the embryos (Table 2.3).

Upon germination, both the total ascorbate pool (Fig. 2.4) and the ability to oxidize GL into AA (Fig. 2.5 A) increased in the embryos of the two embryogenic lines. The restoration of the AA metabolism was more pronounced in the embryos of the (E)WS2 cell culture. After 10 days on the germination medium, the endogenous

Table 2.2. Activities of the redox enzymes of the ascorbate system in the non-embryogenic line (NE)WS and the two embryogenic (E)WS1 and (E)WS2 lines of white spruce during the maturation period. The values are expressed in units ± SE. AAP, ascorbate peroxidase; AFRR, ascorbate free radical reductase; AAO, ascorbate oxidase. [A]1 unit = 1 nmol AA oxidized mg^{-1} protein min^{-1}; [B]1 unit = 1 nmol NADH oxidized mg^{-1} protein min^{-1}. ND, not detected.

Treatment	(NE)WS			(E)WS1			(E)WS2		
	AAP[A]	AFRR[B]	AAO[A]	AAP[A]	AFRR[B]	AAO[A]	AAP[A]	AFRR[B]	AAO[A]
2,4-D (day 4)	228±14	234±22	ND	227±21	295±30	ND	194±10	176±11	ND
2,4-D (day 7)	251±21	244±20	ND	209±17	274±26	ND	206±24	203±19	ND
HF (day 4)	223±31	257±18	ND	190±14	287±22	ND	179±14	172±16	ND
HF (day 7)	243±19	240±15	ND	187±19	285±27	ND	193±20	172±13	ND
ABA liq. (day 4)	224±18	219±16	ND	134±14	298±23	ND	212±17	186±15	ND
ABA liq. (day 7)	225±18	192±21	ND	120±9	277±26	ND	225±21	194±11	ND
ABA sol. (day 7)	174±20	119±9	ND	97±7	234±16	1±0	125±14	185±17	ND
ABA sol. (day 14)	191±12	113±18	ND	77±6	207±20	1±0	118±7	161±14	ND
ABA sol. (day 21)	143±16	128±17	ND	44±3	187±14	2±0	101±9	140±14	ND
ABA sol. (day 28)	126±12	99±7	ND	39±2	180±13	3±0	97±8	132±16	ND
ABA sol. (day 35)	120±10	96±5	ND	33±3	156±16	8±2	73±5	117±7	ND

AA level in the (E)WS2 embryos was higher than that measured in the (E)WS1 embryos (Fig. 2.4). This reflected a higher AA / DHA+AFR ratio observed in the (E)WS2 embryos (Fig. 2.5 B). With the restoration of the AA metabolism a conspicuous increment of the enzymatic activity was observed. particularly in the (E)WS2 embryos. As reported on Table 2.3, after 10 days in germination medium. the specific activities of both AAP and AFRR in the embryos of the (E)WS2 line was twice as high as that observed in the germinating embryos of the other embryogenic line. AAO and DHAR were not detectable at any stage of the PDT and germination (data not shown).

Zygotic embryo maturation and germination

Morphological characteristics of developing seeds

On the first day of collection (June 4. 1999). the seeds were composed of a thin seed coat surrounding the megagametophyte which included two archegonia (Fig. 2.6 A). Embryo development started between June 21 and June 28. Division of the zygote resulted in the formation of a proembryo at the base of the fertilized archegonium. Elongation of the upper tier of cells, which will constitute the suspensor. pushed the developing proembryo into the megagametophytic tissue (Fig. 2.6 B). Further growth of the embryos occurred between July 5 and August 9. Both the suspensor region and the embryo proper. characterized by a well developed protoderm. increased in size (Fig. 2.6 C). Differentiation of both root and shoot apical meristems occurred between July 12 and July 26. and this was soon followed by the emergence of the ring of cotyledons (July 19-August 3) at the shoot apical pole. which demarcated the cotyledonary stage of embryo development (Fig. 2.6 D). Mature embryos. characterized by well developed shoot and root apical meristems (Fig. 2.6 E, F) were visible by August 9. From July 26 to August 30. the moisture content of the seeds declined, as shown by a higher dry weight / fresh weight ratio (data not shown).

Fig. 2.4. Changes in ascorbate (open bars) and dehydroascorbate (solid bars) content during the partial drying treatment (PDT) and germination (germ) of the white spruce somatic embryos characterized by low [(E)WS1] and high [(E)WS2] percentages of conversion. Values are the means $\pm$ SE of three or more experiments.
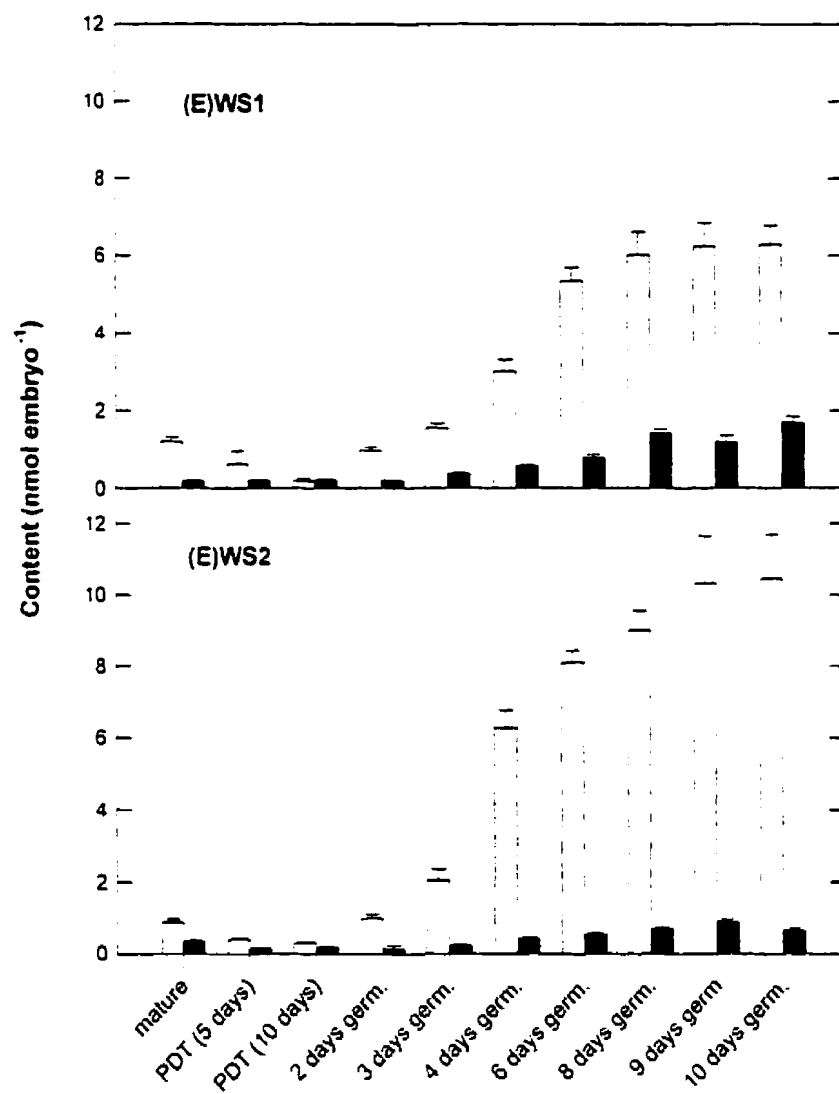
Fig. 2.5. (A) Ability of the white spruce somatic embryos characterized by low [(E)WS1] and high [(E)WS2] percentages of conversion, to generate ascorbate (AA) from its last precursor L-galactono-γ-lactone (GL) during the partial drying treatment (PDT) and germination (germ.). Values are the means ± SE of three or more experiments.

(B) Ratio of the reduced (AA) and oxidized (DHA–AFR) forms within the total ascorbate pool of the white spruce somatic embryos characterized by low [(E)WS1] and high [(E)WS2] percentage of conversion during the partial drying treatment (PDT) and germination (germ.).
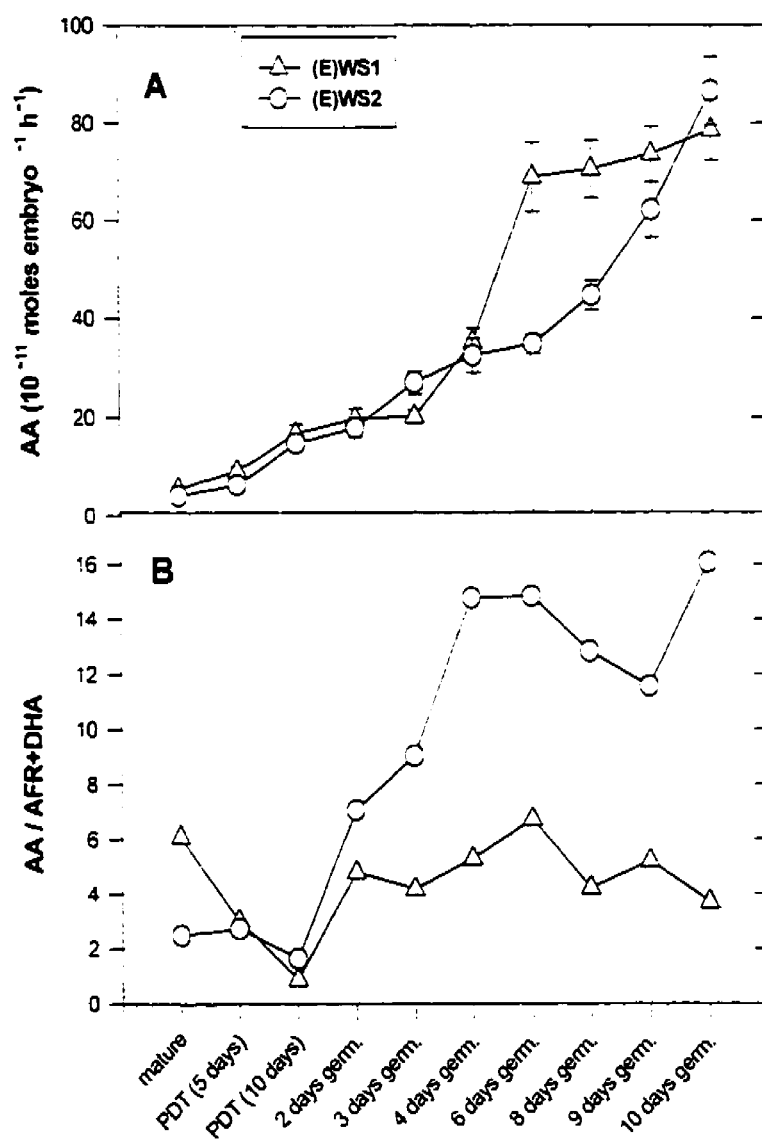
Table 2.3. Activities of the redox enzymes of the AA system of white spruce somatic embryos of the two embryogenic lines (E)WS1 and (E)WS2 during the partial drying treatment (PDT) and germination. The values are expressed in units ± SE. AAP, ascorbate peroxidase; AFRR, ascorbate free radical reductase; [A]1 unit = 1 nmol AA oxidized mg^{-1} protein min^{-1}; [B]1 unit = 1 nmol NADH oxidized mg^{-1} protein min^{-1}.

Treatment	(E)WS1		(E)WS2	
	AAP[A]	AFRR[B]	AAP[A]	AFRR[B]
Mature embryos	48±5	135±10	105±8	168±15
PDT (5 days)	18±1	118±8	45±3	156±12
PDT (10 days)	15±1	84±8	17±2	135±11
Germ. (2 days)	22±2	80±5	60±7	145±13
Germ. (3 days)	44±3	78±5	76±5	164±15
Germ. (4 days)	48±5	86±8	102±10	172±13
Germ. (6 days)	62±5	91±8	138±11	184±18
Germ. (8 days)	68±5	90±7	189±16	211±16
Germ. (9 days)	77±6	87±8	194±20	204±20
Germ. (10 days)	82±9	98±10	212±18	213±18

Fig. 2.6. Micrographs showing the development of white spruce seeds. On June 4.

the seeds were composed of a thin seed coat enclosing the megagametophyte (M)

which included two archegonia (*) (A). A proembryo (arrowhead), subtended by an

elongated suspensor (S), became visible between June 21 and June 28 (B). Upon

further development, both suspensor (S) and embryo proper (arrowhead) increased

in size (C). Differentiation of the apical meristems, followed by the initiation of the

cotyledons (arrowheads), was observed between July 12 and July 26 (D). Mature

somatic embryos were characterized by well developed apical meristems. The

shoot meristem was composed of an apical layer of large, cytoplasmic cells

(arrowhead) and by a sub-apical region (*) where storage product deposition

occurred (E). Large isodiametrical cells (arrowheads), staining less intensely than

the surrounding cells, formed the root meristem (F). Scale bars = 500 μm A: 50 μm

B-F.
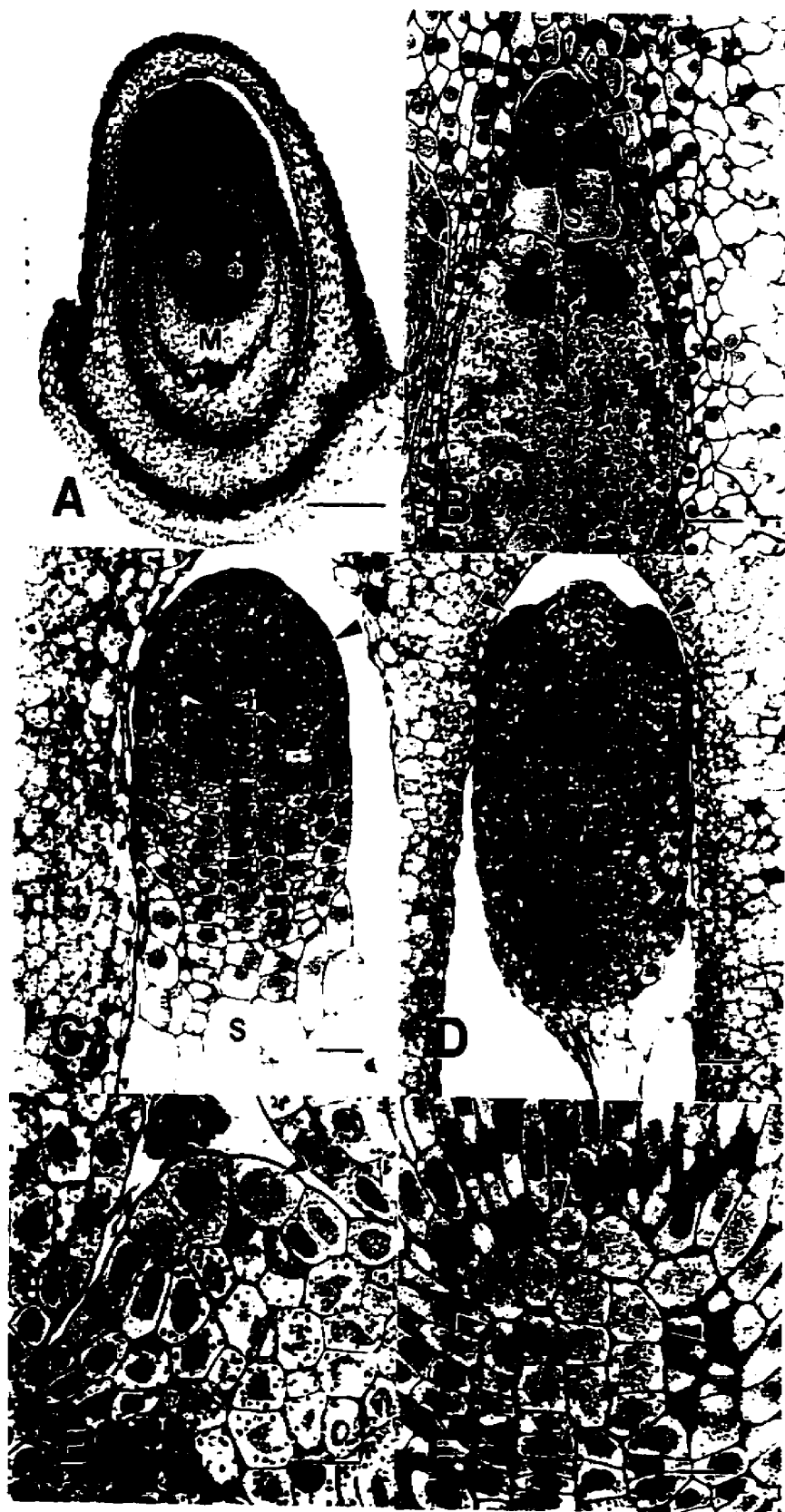
AA metabolism during seed maturation

During the early stages of white spruce seed development (June 4-July 12) the total ascorbate pool (AA+DHA+AFR) was lower than 10 nmol seed^{-1}. Within the total ascorbate pool. similar levels of AA and DHA+AFR were measured, and the AA / DHA+AFR ratio was close to 1 (Fig. 2.7). Upon further development. the amount of endogenous AA sharply increased. reaching the highest peak on August 16. after which it declined. Similar increases were also observed for the AA / DHA+AFR ratio (Fig. 2. 7) and the ability of seeds to produce AA de novo (Fig. 2.8). Stored seeds were devoid of AA. and only a small amount of DHA+AFR was present (Fig. 2.7). Among the enzymes of AA metabolism. the activities of ascorbate peroxidase (AAP) and ascorbate free radical reductase (AFRR) were high during the initial phases of seed maturation and declined after July 12. In stored seeds only the activities of AFRR and DHAR were detected (Table 2.4).

AA metabolism in dissected embryos and megagametophytes of germinating seeds

AA metabolism was investigated in dissected embryos and separated megagametophytes of germinating seeds. Both dry zygotic embryos and megagametophytes were devoid of AA. and they contained only small traces of DHA+AFR (Fig. 2.9). Upon germination. both AA and DHA+AFR increased. especially in the embryos. After 12 days of germination. the level of AA was almost double that of DHA+AFR in both embryos and megagametophytes (Fig. 2.9). Activities of AFRR and DHAR were detected in both dry embryos and megagametophytes (Table 2.5). Upon germination. the activity of AFRR increased. whereas that of DHAR decreased. especially in the embryos. Ascorbate peroxidase (AAP) was absent in dry embryos and megagametophytes. and its activity was only detected after 3 days of germination (Table 2.5).

Fig. 2.7. Changes in ascorbate (open bars) and dehydroascorbate (solid bars)

content and AA DHA-AFR ratio during the maturation of white spruce seeds.

Values are the means = SE of three or more experiments.
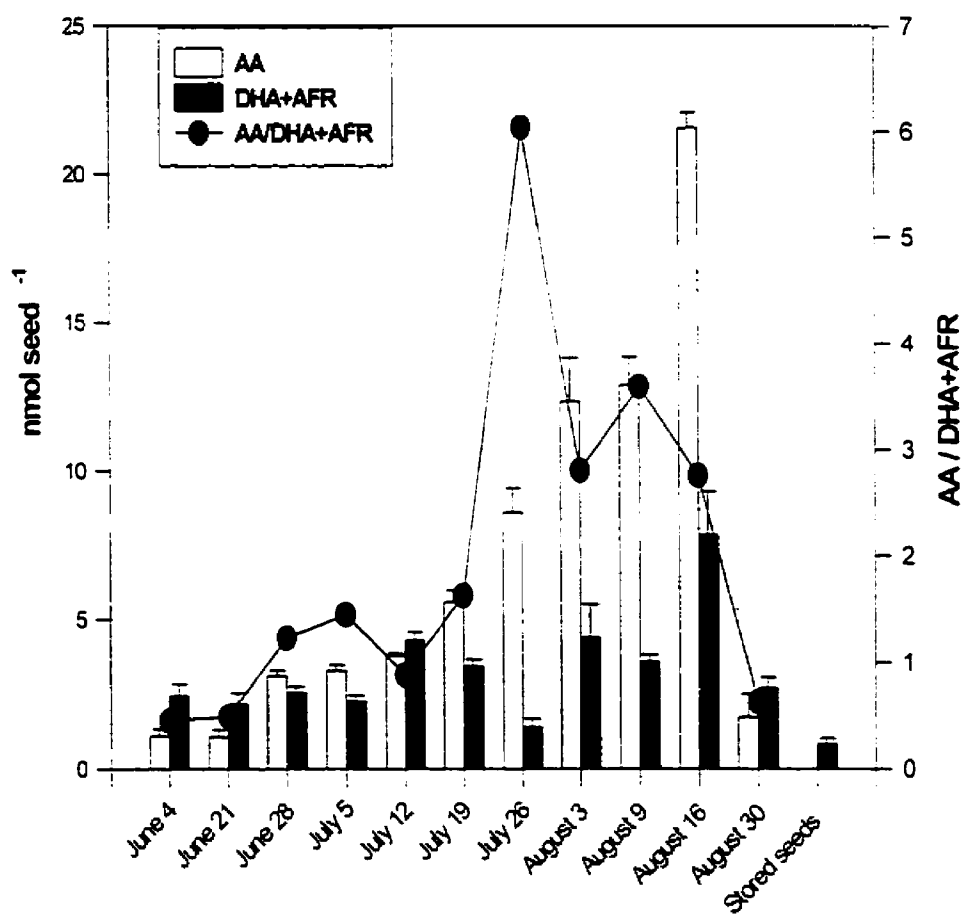
Fig. 2.8. Ability of maturing white spruce seeds to generate ascorbate (AA) from its last precursor L-galactono-γ-lactone (GL). Values are the means $\pm$ SE of three or more experiments.
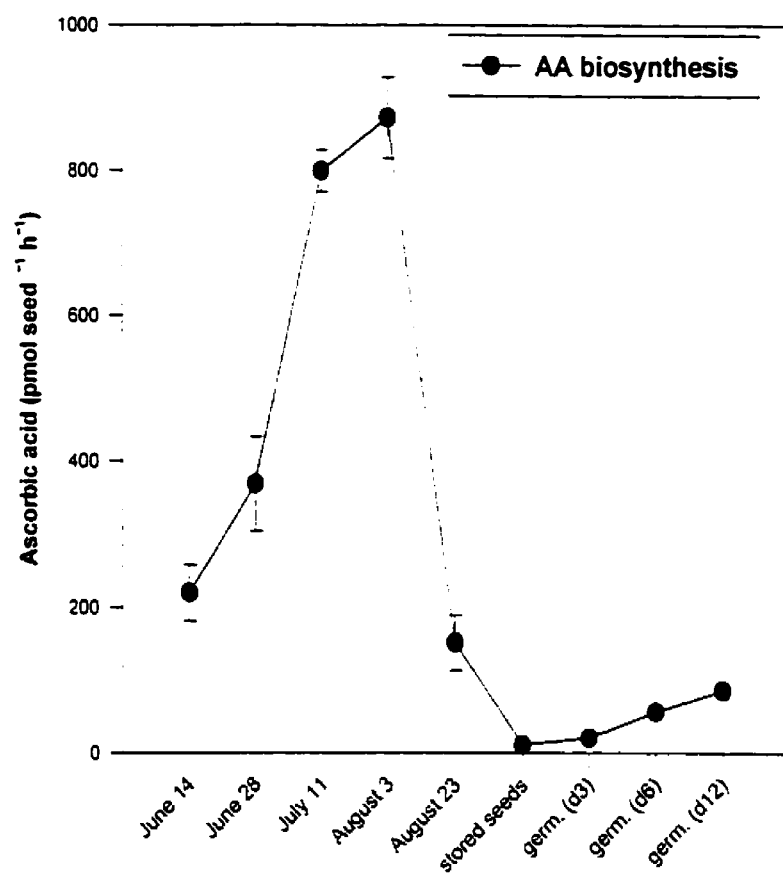
Table 2.4. Activities of the redox enzymes of the AA system in maturing white spruce seeds. The values are expressed in units ± SE. AAP, ascorbate peroxidase; AFRR, ascorbate free radical reductase; [A]1 unit = 1 nmol AA oxidized seed^{-1} min^{-1}; [B]1 unit = 1 nmol NADH oxidized seed^{-1} min^{-1}; [C] 1 nmol AA reduced seed^{-1} min^{-1}. ND, not detected.

Date of collection	AAP[A]	AFRR[B]	DHAR[C]	AAO[A]
June 14	20.0±5.1	12.8±1.6	0.9±0.1	0.1±0.0
June 21	25.3±4.6	14.1±0.9	1.1±0.4	ND
June 28	20.6±2.4	15.6±1.0	0.8±0.0	ND
July 5	27.2±1.4	20.6±1.0	0.7±0.4	ND
July 12	31.2±1.6	10.5±0.9	0.7±0.1	ND
July 19	9.0±0.4	6.2±0.5	0.6±0.0	0.1±0.0
July 26	7.9±0.8	2.6±0.3	0.9±0.3	0.1±0.0
August 3	4.2±0.5	1.3±0.2	1.2±0.3	ND
August 16	3.0±0.1	2.2±0.4	0.8±0.0	ND
August 30	0.8±0.2	2.3±0.3	0.9±0.3	ND
Stored seeds	ND	2.3±0.2	0.6±0.4	ND

Fig. 2.9. Changes in ascorbate (open bars) and dehydroascorbate (solid bars)

content and of zygotic embryos (**A**) and megagametophytes (**B**) dissected from

germinating (germ.) seeds. Values are the means ± SE of three or more experiments

pmol / embryo or megagametophyte

A

B

Stored seeds   3 days germ.   6 days germ.   12 days germ.
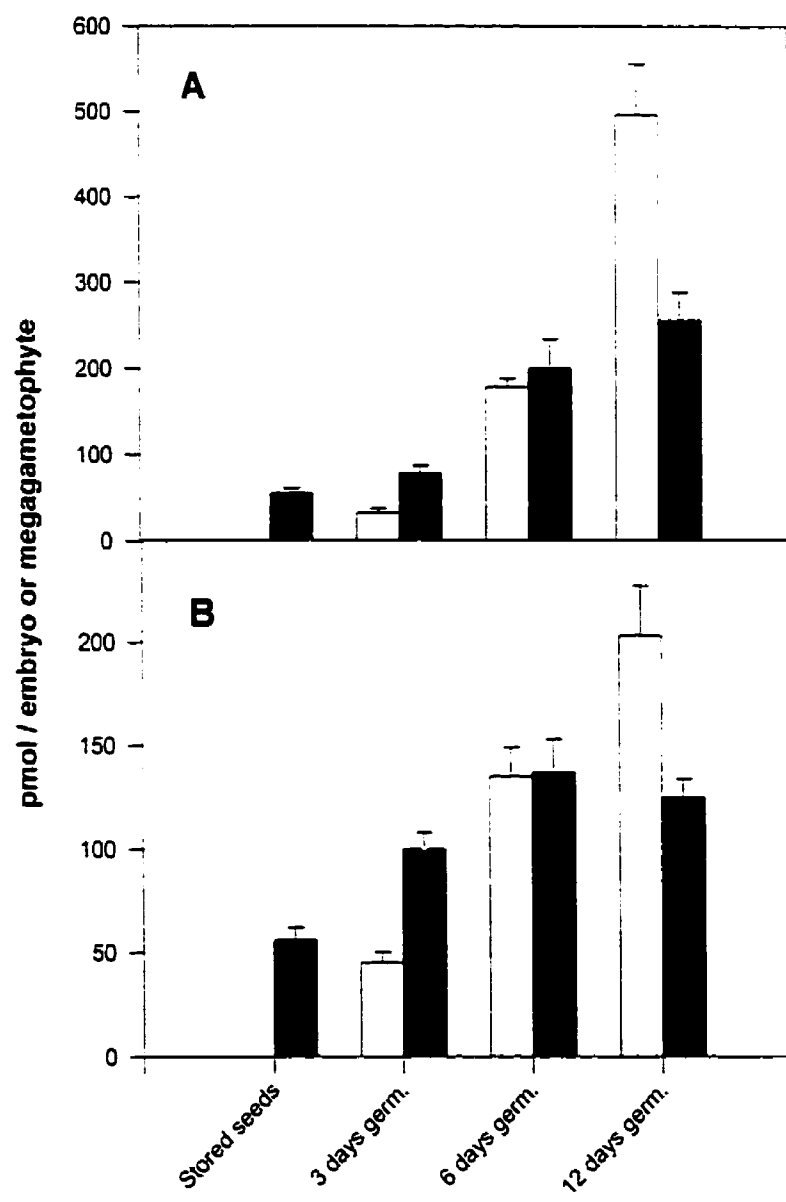
Table 2.5. Activities of the redox enzymes of the AA system in zygotic embryos and megagametophytes (Megagam.) of germinating seeds. The values are expressed in units ± SE. AAP, ascorbate peroxidase; AFRR, ascorbate free radical reductase; DHAR, dehydroascorbate reductase; AAO, ascorbate oxidase. [a] 1 unit = 1 nmol AA oxidized embryo or megagametophyte^{-1} min^{-1}; [b] 1 unit = 1 nmol AA oxidized embryo or megagametophyte^{-1} min^{-1}; [c] 1 nmol AA reduced embryo or megagametophyte^{-1} min^{-1}; [d] 1 nmol NADH oxidized embryo or megagametophyte^{-1} min^{-1}. ND, not detected.

Days of germination	AAP[a]		AFRR[b]		DHAR[c]		AAO[d]	
	Embryo	Megagam.	Embryo	Megagam.	Embryos	Megagam.	Embryo	Megagam.
Day 0	ND	ND	1.4±0.5	2.1±0.1	0.6±5.1	0.1±0.0	ND	ND
Day 3	0.5±0.0	0.1±0.0	1.8±0.2	1.9±0.2	0.5±0.1	0.1±0.1	0.1±0.1	ND
Day 6	3.4±0.2	1.2±0.4	3.2±0.4	2.2±0.1	0.3±0.0	ND	0.2±0.1	ND
Day 12	6.0±0.7	1.7±0.3	4.7±0.3	3.4±0.7	0.1±0.0	0.1±0.1	ND	ND

Discussion

In order to determine whether (1) differences in AA metabolism occur during zygotic and somatic embryogenesis and (2) alterations in AA synthesis and utilization correlate with the ability of white spruce cultured cells to produce good quality somatic embryos, AA metabolism was investigated in both zygotic and somatic embryos. For this purpose, three distinct white spruce cell lines were utilized: a non-embryogenic line (NE)WS, unable to generate mature embryos, and two embryogenic lines with low (E)WS1 and high (E)WS2 percentage of embryo germination.

Somatic embryo maturation and germination

AA metabolism during somatic embryo maturation

Only small differences in AA metabolism were observed among the three lines in the maintenance (2,4-D) medium. The fact that the total acorbate pool (AA+AFR+DHA) was similar in all the three lines, despite their different increases in fresh and dry weight in the maintenance medium (data not shown), indicates that AA might be involved in other cellular processes besides cell division. The high enzymatic activity observed in the maintenance medium (Table 2.2), especially that of AAP which uses AA as a substrate, suggests that AA may be required for the removal of reactive oxygen species, as well as for promotion of cell wall plasticity. Besides being involved in decreasing the toxic levels of hydrogen peroxide, in concert with catalases, AAP has also been found to promote cell wall plasticity by decreasing the availability of H_2O_2, utilized by cell wall peroxidases in cross linking matrix polymers (De Gara et al., 1996). This hypothesis is also supported by kinetic studies showing the higher affinity of AAP for H_2O_2 when compared to other peroxidases (see De Gara and Tommasi, 1999). Cleavage polyembryony, a common phenomenon during white spruce somatic embryogenesis necessary for the perpetuation of the culture, may be facilitated by all those processes

promoting cell wall plasticity.

Among the changes in AA metabolism observed in the three cultures upon transfer onto the maturation medium, the alteration of the AA / DHA+AFR ratio appears to be the most prominent. The switch of this ratio towards the reduced state (AA) correlates with the induction of somatic embryo development in the two embryogenic lines (E)WS1 and (E)WS2. A similar alteration within the total ascorbate pool was also observed by previous investigations. Arrigoni et al. (1992) reported a high AA / DHA+AFR ratio during the early stages of *Vicia faba* seed development, characterized by an active metabolism and a high mitotic activity. During the subsequent phases of seed maturation, characterized by cell elongation, a gradual decline of the same ratio occurred. The observed increment of the AA / DHA+AFR ratio may therefore be required for somatic embryo growth. Firstly, no increment of the ratio occurred in the non-embryogenic culture upon transfer onto the maturation medium (Fig. 2.3 B). In the (NE)WS line, in fact, a developmental block seems to occur during the early phases of embryo maturation. Secondly, the different increment of the ratio in the three cultures 7 days after transfer onto the ABA-containing medium (ABA liq. [day 7]), strongly correlates with the different stages of development of the maturing embryos. A higher AA / DHA+AFR ratio was observed in the (E)WS1 cell line that presents structurally organized globular-shaped embryos with a well defined protoderm layer and a conspicuous root cap (Fig. 2.1 E). In the (E)WS2 line, the poorly developed embryogenic heads without any sign of morphological differentiation (Fig. 2.1 J) correlate with a lower AA / DHA+AFR ratio. However, during the second half of the maturation period, when embryo development occurs, a sharp switch towards the reduced state was observed in the ratio. In the non-embryogenic line, where embryo development does not occur (Fig. 2. 1 C), the AA / DHA+AFR ratio remains close to 1.

Although in vitro manipulations aimed at increasing the AA / DHA+AFR ratio in the non-embryogenic line have failed to promote embryo formation (data not shown), the high AA level within the total ascorbate pool, observed during somatic embryo maturation in the two embryogenic lines, may be required to carry out fundamental

processes such as cell division. As indicated by several authors, in fact, a large amount of AA is present in tissue during intense growth. De Gara et al. (1996) reported that in pea stem the content of AA is high in the meristematic region and it gradually declines in older tissues. Similarly, exogenous applications of AA have been found to induce cell divisions in several systems such as *Allium cepa* root (Innocenti et al., 1990), *Zea mays* root (Kerk and Feldmann, 1995), and cambial cells of *Lupinus albus* root (Arrigoni et al., 1997). Furthermore, experimental reduction of the endogenous AA levels by applications of lycorine, an inhibitor of the last enzyme of the de-novo AA biosynthesis, inhibits cell division (De Leo et al., 1973).

Together with the beneficial effects of AA, a low level of DHA within the total ascorbate pool may be required for white spruce embryo maturation. Deleterious effects of DHA have been recently observed by Morell et al. (1997). Furthermore, De Gara and Tommasi (1999) reported a reduced growth of tobacco cells cultured in the presence of DHA. Once the level of DHA was lowered to the control level cell growth resumed. Another interesting point emerging from our results is the increased capability to synthesize AA de novo, observed in the two embryogenic lines upon transfer onto the maturation medium. This enhanced ability to oxidize GL into AA, rather than the AFRR activity which is similar between the (NE)WS and (E)WS2 lines, may be responsible for the increment of the AA / DHA+AFR ratio. The fact that maturing somatic embryos are endowed with the ability to produce AA de novo suggests that differences in AA metabolism exist between zygotic and somatic embryogenesis. In *Vicia faba* seeds, the large amount of AA needed during the early stages of development is provided by the parent plant, since the ability to synthesize AA is only acquired by the seeds as desiccation proceeds (Arrigoni et al., 1992). The absence of the parent tissue in somatic embryos imposes metabolic adjustments necessary to carry out all those cellular processes that in the zygotic counterparts are under the control of the parent tissue. That morphological and physiological differences exist between somatic and zygotic embryogenesis is well documented in literature (Joy et al., 1991b; Kong and Yeung, 1992; Misra et al., 1993).

In *Vicia faba* seeds, the AA / DHA+AFR ratio slowly declined during the late stages of embryo maturation. when cell elongation occurs. This switch of the total AA pool towards the oxidative state does not occur in white spruce somatic embryos. since a high AA / DHA+AFR ratio is present at the end of the maturation period (Fig. 2.3 B). This difference in pattern may be ascribed to the fact that limited cell expansion occurs during white spruce somatic embryogenesis.

AA metabolism during the partial drying treatment (PDT) and germination

During the partial drying treatment, the overall AA metabolism slowly declines. The decrease in the total AA pool is concomitant with a decline of its redox enzymes. It is worth noting, however, that the ability of the partially dried embryos to oxidize GL into AA increases. Similar changes in the AA metabolism were also reported during the desiccation period that characterizes the late stages of seed maturation. The seeds of several angiosperms. in fact. reach maturity in a dehydrated state and they are devoid of AA. even though they are endowed with the ascorbate biosynthetic system (Arrigoni et al.. 1992) necessary to meet the large demand of AA during the early stages of germination. The limited presence of AA observed in white spruce somatic embryos after the PDT may be ascribed to the higher water content (about 65%) still present in the partially dried embryos when compared to that of desiccated seeds. The decrease of endogenous AA levels in the embryos parallels the decline of the AAP specific activity. It is well documented in the literature that the activity of AAP is tightly regulated by the availability of AA in the system. The absence of AAP activity has been found in those systems devoid of AA such as dried *Vicia faba* seeds (Arrigoni et al.. 1992) and mature *Avena sativa* embryos (Tommasi and De Gara. 1990).

A large amount of AA was produced by the embryos of the two embryogenic lines upon transfer onto the germination medium. The involvement of this metabolite in cell division (Liso et al.. 1984; Citterio et al.. 1994) and cell elongation (Cordoba-Pedregosa et al. 1996; Arrigoni et al.. 1997) processes has been extensively investigated. The high

levels of AAP observed in germinating embryos also confirms that the activity of this enzyme may be regulated by the availability of AA. With the recovery of the oxidative metabolism, high levels of AAP are necessary to eliminate reactive oxygen species such as hydrogen peroxide. The key role played by AAP in the removal of H_2O_2 has been extensively discussed in the literature (see Smimoff, 1996; Noctor and Foyer, 1998). Therefore, a failure or a delayed restoration of the AA metabolism upon germination may result in a toxic accumulation of hydrogen peroxide which would seriously compromise the viability of the embryos. This hypothesis is supported by the results that show the existence of a positive correlation between embryo germination and restoration of AA metabolism. Upon transfer onto the germination medium, both the total ascorbate pool and the specific activity of the AA redox enzymes are higher in the embryos of the (E)WS2 cell line characterized by a high percentage of embryo germination. Moreover, the higher AA / DHA+AFR ratio observed in these embryos, also suggests that a large amount of AA is available during germination.

Zygotic embryo maturation and germination

During the initial stages of white spruce seed development, the total ascorbate pool (AA+DHA+AFR) was low (Fig. 2.7), despite the high activity of the AA-redox enzymes, especially AAP (Table 2.4). As mentioned for the somatic counterparts, the high activity of this enzyme in developing zygotic embryos may be required for removal of reactive oxygen species, as well as promotion of cell wall plasticity required for suspensor elongation (Fig. 2.6 B) and growth of the embryo proper within the megagametophytic tissue (Fig. 2.6 C). The observation that AAP activity decreases after July 12, when the activity of guaiacol peroxidase, one of the major enzymes involved in cell wall crosslinking, increases (data not shown), supports this notion. The increment of the AA / DHA+AFR ratio during the most intense growth of the zygotic embryos indicates that a large availability of AA is needed to sustain the growth of the developing embryo. A similar result was also found during somatic embryogenesis (Fig. 2.3 B) and

during *Vicia faba* seed development (Arrigoni et al.. 1992).

During the later stages of seed development. characterized by a loss of moisture content. both the total ascorbate pool (AA+AFR+DHA) (Fig. 2.7) and the ability to oxidize GL to AA (Fig. 2.8) declined. In contrast to partially dried embryos. dried seeds do not dispose of an efficient system to generate AA de novo. Furthermore. DHAR. not detected at any stages of somatic embryogenesis, was found in dried seeds. together with AFRR (Table 2.4). Residual activity of these AA-recycling enzymes in dry seeds may represent a strategy for providing the embryos with sufficient AA during the initial stages of germination. prior to the reactivation of the AA de novo machinery. Several studies have in fact revealed the presence of these two enzymes and their substrates (DHA and AFR) in dried embryos of many plant species (see Tommasi et al.. 1999). The fact that in germinating zygotic embryos the ability to oxidize GL to AA slowly increases. whereas DHAR activity decreases. supports this notion. As germination progresses. both the total ascorbate pool and the activities of AAP and AFRR increase. especially in the dissected embryos. Restoration of the overall AA metabolism upon imbibition. also observed in somatic embryos. is in fact required for carrying out many fundamental cellular processes. including cell division and elongation. In tissue not subjected to growth. i.e. megagametophytic tissue, such a restoration of AA metabolism is limited.

Conclusions

Alterations of AA metabolism are very similar during the initial stages of somatic and zygotic embryo development. The increased ability of the developing embryos to generate AA de novo. together with an increase in the AA / DHA+AFR ratio seems to be required for initiation of the in vivo and in vitro embryogenic processes. Significant differences in AA metabolism. were only observed at later stages of development, during the natural desiccation period in zygotic embryos and the imposed partial drying

treatment (PDT) in somatic embryos. The presence of the AA-recycling enzyme DHAR in dried seeds may be necessary for a prompt conversion of DHA to AA at the onset of germination, prior to the reactivation of the AA de novo machinery. The absence of DHAR observed in somatic embryos at the end of the PDT may be due to the fact that partially dried embryos have the ability to produce AA de novo. Such differences in metabolism may be partially ascribed to the different level of water stress experienced by somatic and zygotic embryos.

Alterations in AA synthesis were also found to correlate with the embryogenic potential of different white spruce cell cultures. The ability of cultured cells to metabolically alter the AA / DHA+AFR ratio by increasing the AA de novo biosynthesis seems to correlate with the initiation of embryo development. Such changes in metabolism, in fact, were not observed in the non-embryogenic line. Furthermore, a positive correlation between the AA / DHA+AFR ratio and the growth of the developing embryos was also observed. The reactivation of AA metabolism at germination seems to be important for successful somatic embryo germination and conversion into viable plantlets. Upon imbibition, in fact, both the total ascorbate pool and the activities of major enzymes of the AA metabolism were higher in the embryos of the (E)WS2 line, which are characterized by a higher germination and conversion frequencies. This finding clearly suggests that experimental manipulations of the endogenous AA level may be utilized for improving the post-embryonic growth of embryos with lower conversion frequencies.

CHAPTER THREE

Effects of exogenous applications of ascorbic acid on white spruce somatic embryo conversion

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Introduction

The ability of white spruce somatic embryos to undergo successful germination and conversion is dependent upon the reactivation of the apical meristems. Structural studies conducted by Kong and Yeung (1992) have revealed that those embryos that fail to germinate are characterized by poorly organized apical meristems, often disrupted by the presence of intercellular air spaces. Upon germination, cells within these meristems stop dividing, and this results in meristem abortion and termination of seedling growth. As mentioned in Chapter 1, the poor post-embryonic performance, observed in many lines of white spruce somatic embryos, represents a major obstacle for a more extensive utilization of somatic embryogenesis for large scale propagation. Therefore, manipulations of culture conditions resulting in promotion of mitotic activity at germination and embryo conversion would have a profound impact on the biotechnology industry.

In the last few years, a lot of attention has been directed towards the involvement of ascorbic acid (AA) in cell division (see Smirnoff, 1996). From several studies, including that reported in Chapter 2, it emerges that AA plays a key role during plant growth, as a positive correlation between endogenous AA level and cell proliferation seems to exist. In pea stem, for example, the AA content is high in the cells of the shoot apical meristem, characterized by active mitotic activity, and it gradually declines in more differentiated tissues (De Gara and Tommasi, 1999). A similar correlation was also reported in corn roots, where the endogenous AA level of cells within the quiescent centre was significantly lower than that measured in the adjacent cells, undergoing intense proliferation (Kerk and Feldman, 1995). Experimental manipulations of the

endogenous AA content further substantiated these findings. Increased levels of cellular AA induced cell division in several systems, including *Zea mays* and *Allium cepa* roots (Liso et al., 1984; Kerk and Feldmann, 1995) and *Lupinus albus* seedlings (Arrigoni et al., 1997), possibly by inducing the progression of cells from G1 to S (Liso et al., 1984). Similarly, a reduction in endogenous AA content by lycorine, an inhibitor of the de novo AA biosynthesis, was found to reduce cell division in pea and onion roots (Citterio et al., 1994; Cordoba-Pedregosa et al., 1996). Thus, it is not surprising that AA has been often utilized in in vitro systems where it promotes growth. Besides stimulating cell division in tobacco cultured cells (de Pinto et al., 1999), exogenous applications of AA have been found to induce shoot formation via organogenesis in tobacco callus (Joy et al., 1988).

In light of these findings, the main objective of this chapter was to test the hypothesis that exogenous applications of AA may increase the conversion frequency of white spruce somatic embryos by stimulating cell division. This was carried out by culturing the embryos of the (E)WS1 line, characterized by a lower percentage of conversion and endogenous AA content compared to those of the (E)WS2 cell line (see Chapter 2), in a germination medium supplemented with AA. Conversion frequency tests and structural studies were conducted in order to determine the effects of AA treatments on embryo conversion and meristem reactivation.

Materials and Methods

Embryogenic tissue induction and maintenance

This was achieved as described in Chapter 2

Embryo maturation and partial drying treatment

This was carried out as reported in Chapter 2.

Embryo germination and AA applications

After the partial drying treatment the embryos were transferred onto the germination medium (half strength AE containing 1% sucrose, without growth regulators) supplemented with different concentrations of ascorbic acid (1. 10. 100. and 1000 µM). The ascorbate solutions (pH. 5.8) were added to the medium by sterile filtration. The external morphology of the embryos was examined over a period of three months and the number of embryos with root only, shoot only, and root and shoot was recorded.

Statistical analyses

Statistical analysis was carried out as described in Chapter 2.

Light microscopy

Tissue preparation and staining procedures have been outlined in Chapter 2.

Results

The effects of AA applications on embryo conversion

In both control and AA-treated embryos, germination (root emergence) occurred between day 5 and day 10. A terminal shoot bud was observed 15-20 days after the embryos were transferred onto the gemination medium. In the control. root and shoot emergence was only observed in 33.5% of the embryos examined. The remaining embryos were unable to undergo normal conversion either for lack of shoot. root. or both (Fig. 3.1). Of the 220 control embryos. 64% were able to produce a normal root. whereas only 47 % were able to produce new leaves (Table 3.1).

The effect of exogenous applications of ascorbic acid on shoot and root

emergence was examined by supplementing the gemination medium with four different levels of AA (1. 10. 100. and 1000 μM). Concentrations of AA ranging from 1-100 μM were found to enhance somatic embryo conversion (i.e. root and shoot emergence) in an almost linear fashion (Fig. 3.1). Analysis of variance indicated that the results from the 100 μM AA treatment were significantly different from those of the control. A higher concentration of AA (1000 μM) was found to be deleterious for embryo conversion (Fig. 3.1). Only 12% of the embryos in this treatment were able to undergo normal root and shoot conversion. Treatments between 100 and 1000 μM of AA were not tested. as the major objective of this study was not to find the optimal AA concentration. but rather to demonstrate the requirement for AA during embryo germination.

When the effects of AA on root and shoot growth were examined independently from one another. it was clear that AA had a much more pronounced effect on shoot growth than on root emergence. With increasing concentrations of AA. the percentage of embryos able to produce new leaf primordia increased from 47% (control) to 79% in the embryos treated with 100 μM AA. Statistical analysis showed that the results from this treatment were significantly different from those of the control. A similar increase. but with a less dramatic effect. was also observed on root growth. Ascorbic acid (100 μM) increased the percentage of embryos able to produce roots from 64% (control) to 74 % (Table 3.1). However. statistical analysis indicated that there was no significant differences in root emergence between the AA-treated embryos (1-100 mM) and the control.

Differences were observed when the external morphology of these embryos was examined. When compared to the control embryos (Fig. 3.2 A-C). AA-treated embryos were visibly larger. New leaves continued to be formed from the shoot apical meristem (Fig. 3.2 D). Furthermore. the embryos were characterized by dark green leaves in contrast to the pale green leaves observed in the control embryos. When the converted AA-treated embryos were allowed to develop further in the greenhouse, plantlets developed normally (Figs 3.2 E).

Fig. 3.1. The effect of ascorbic acid on white spruce somatic embryo conversion in the (E)WS1 line. Each value represents the mean ± SE of at least three replicate experiments with 60-80 embryos per treatment in each experiment.
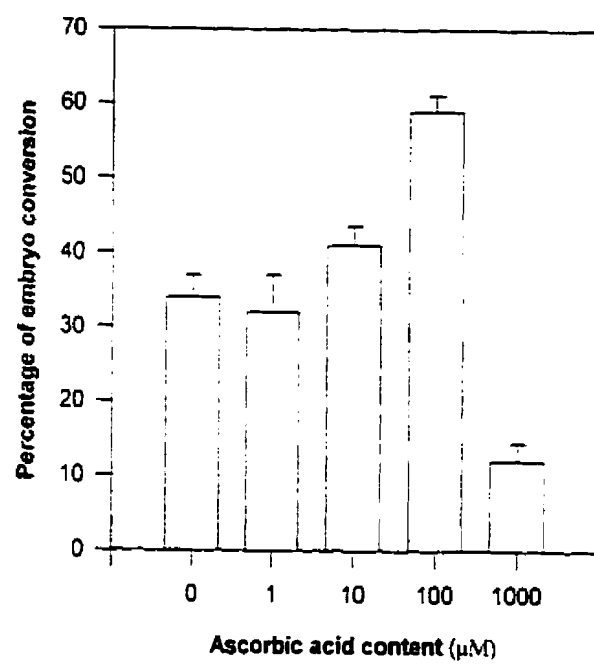
Table 3.1. The effect of ascorbic acid (AA) on shoot and root growth of white spruce somatic embryos in the (E)WS1 line. R. root growth only: S. shoot growth only: N. no root or shoot growth.

Treatment	R(%)	S(%)	N(%)
Control	64.13±5.71	46.75±5.06	20.33±2.90
AA(1 μM)	66.40±3.01	52.50±5.03	12.52±1.80
AA(10 μM)	66.53±1.20	61.08±4.74	11.48±2.25
AA(100 μM)	73.92±4.05	78.70±5.63	5.38±1.52
AA(1000 μM)	39.57±1.15	31.55±2.30	40.43±4.58

Fig. 3.2. The morphology of (E)WS1 white spruce somatic embryos in the presence or absence of ascorbic acid (AA). Among the control embryos, three different populations could be observed. Converting embryos, characterized by an elongated root and a functional shoot, as denoted by the presence of new leaf primordia (A), non-converting embryos with only shoot growth (B), and non-converting embryos with only root growth (C). Many of the AA-treated embryos were able to convert, and they were generally bigger than their control counterparts (D). Upon further growth, such embryos developed into normal plantlets (about 5 months old) (E). Scale bars = 1 cm.

A B C D
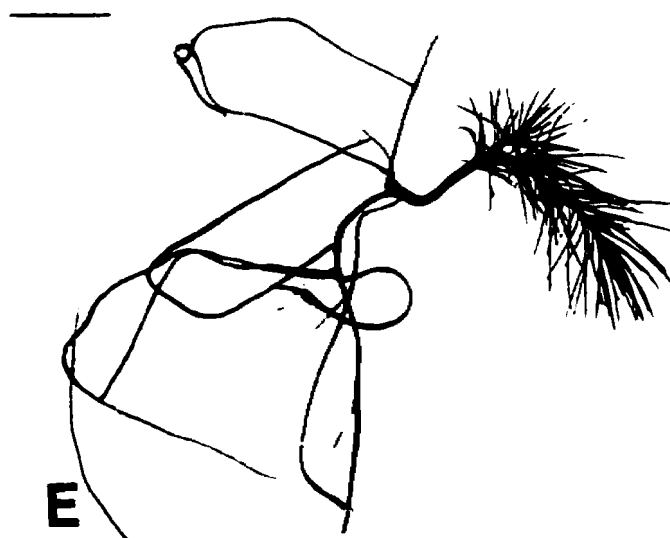
E

Effects of AA on the organization of the shoot apical meristems

Apical meristem organization after the partial drying treatment

Three different shoot apical meristem organizations could be found among the somatic embryo population. In some embryos, the shoot meristems appeared normal. These shoot meristems had a distinct apical layer, characterized by cytoplasmic cells. This apical layer was subtended by sub-epidermal cells with small intercellular air spaces. The sub-epidermal cells accumulated large amount of storage products (Fig. 3.3 A). In other embryos, the organization of the sub-epidermal cells was disrupted by the presence of intercellular air spaces (Fig. 3.3 B). In some instances, these air spaces caused complete separation of the sub-epidermal cells which resulted in poor meristem organization (Fig. 3.3 C).

Embryos in the control germination medium

In control embryos with well organized shoot meristems two different patterns of development were observed at germination. In most of the embryos, anticlinal cell divisions in the apical layer of the shoot meristem could be found 2 days after placement on the control germination medium (Fig. 3.4 A). After 4 days, several mitotic figures were observed in both apical and sub-apical layers of the meristem (Fig. 3.4 B). Mitotic activity from the lateral region of the meristem resulted in the formation of leaf primordia, which became visible around day 10 (Fig. 3.4 C). During the following days, a well organized shoot primordium characterized by elongated leaf primordia became visible (Fig. 3.4 D).

Some control embryos with well organized meristems failed to convert at germination. Careful examination of sections through the shoot pole of these embryos indicated that although mitotic activity was observed at day 2, the apical cells started to accumulate phenolics (Fig. 3.5 A).

Fig. 3.3 Different shoot meristem organizations observed in the somatic embryos of the (E)WS1 line after the partial drying treatment. In well organized meristems, a distinct apical layer of cytoplasmic cells (arrowhead) could be seen. This layer of cells was subtended by a sub-apical region with small intercellular spaces (*) (**A**). In some meristems, the sub-apical region was disrupted by prominent intercellular air spaces (*) (**B**). Increasing formation of these air spaces (*) caused a complete separation of the sub-apical cells, resulting in abnormal meristem structure (**C**). All scale bars = 50 μm.

Upon further development, accumulation of phenolics continued in the cells of the apical and sub-apical layers of the shoot meristem (Fig. 3.5 B). Large vacuoles appeared in the sub-apical cells, and this was soon followed by cessation of cell division, which resulted in meristem abortion (Fig. 3.5 C). In some meristems, cessation of cell division was a later event, as dome-shaped shoot primordia were formed. However, the continual deposition of phenolics in the cells of these meristems, as well as increased vacuolation, prevented any further growth (Fig. 3.5 D).

For those embryos characterized by poorly organized meristems (Fig. 3.3 B. C), pronounced accumulation of phenolics was commonly observed after 4 days in germination (Fig. 3.6 A). During the following days of germination, accumulation of phenolics increased, especially in the apical cells (Fig. 3.6 B). Mitotic activity was seldom detected in these meristems, which were not able to undergo further development (Fig. 3.6 C).

Accumulation of phenolic compounds in well organized and poorly organized meristems of control embryos was evident after staining the sections with the toluidine blue O stain (Fig. 3.7).

Embryos in AA containing medium

Exogenous applications of AA in the germination medium induced abnormal meristems (Fig. 3.3 B. C) to undergo further development, resulting in the formation of a new "normal" shoot primordium. Cell division was observed in these meristems after 2 days in germination, despite the presence of large intercellular air spaces (Fig. 3.8 A). Continual mitotic activity of some apical cells resulted in the formation of a dome-shaped primordium emerging from the shoot pole (Fig. 3.8 B). After 9 days in the germination medium, organized shoot primordia, composed by densely stained cytoplasmic cells, were subtended by vacuolated cells separated by large intercellular air spaces. A pronounced accumulation of phenolics, similar to that described for the control counterparts, was seldom observed in AA-treated embryos, especially in the developing shoot primordia

(Fig. 3.8 C). The formation of well developed leaf primordia, observed at day 16, indicated that these primordia were fully functional (Fig, 3.8 D).

In some instances, although the shoot apical meristem remained non-functional, AA applications resulted in the activation of cells at the base of the cotyledons near the apical meristem (Fig. 3.9 A). These cells were able to divide further and gave rise to meristematic regions originating from the base of the cotyledons (Fig. 3.9 B). Continuation of mitotic activity along the base of the cotyledons resulted in several shoot primordia, easily discernible by the presence of densely stained cytoplasmic cells (Fig. 3.9 C). These shoot primordia were able to function as normal meristems, as indicated by their ability to generate leaf primordia (Fig. 3.9 D).

Discussion

The failure to resume mitotic activity in the meristems is responsible for the poor performance of white spruce somatic embryos at germination (Kong and Yeung. 1992). Data presented here indicate that exogenous applications of AA enhance the postembryonic performance of white spruce somatic embryos of the (E)WS1 line, by increasing the percentage of embryo conversion from 34% (control) to 59% (100 μM AA) (Fig. 3.1). Furthermore, the linear dosage-response effect of AA on embryo conversion clearly suggests that the endogenous AA level may be limiting the post-embryonic performances of the embryos. As reported in Chapter 2, the endogenous AA levels of the (E)WS1 embryos was lower than that measured in the embryos of the (E)WS2 line, characterized by a higher conversion frequency. These data are in agreement with previous work indicating the requirement for AA during periods of intense growth (discussed in Chapter 2). Experimental manipulations that alter the cellular AA content, in fact, have been found to affect the growth in many systems, including tobacco cultured cells (de Pinto et al., 1999), corn and onion root cells (Liso et al., 1984; 1988; Kerk and Feldman, 1995), and *Lupinus albus* seedlings (Arrigoni et al., 1997). In onion roots, for example, a decrease in the AA level caused by addition of

Fig. 3.4. Morphology of a "well organized" shoot apical meristem of a germinating control embryo of the (E)WS1 line. Anticlinal divisions (arrowhead) in the apical layer of the meristem were observed at day 2 (A). At day 4, mitotic activity increased in both apical and sub-apical layers. The meristem assumed a dome-shaped appearance (B). Continual mitotic activity, especially in the peripheral region of the meristem resulted in the emergence of leaf primordia (arrowheads) (C). After 14 days in germination medium, a functional shoot primordium, characterized by elongated leaf primordia was observed (D). All scale bars = 40 μm.
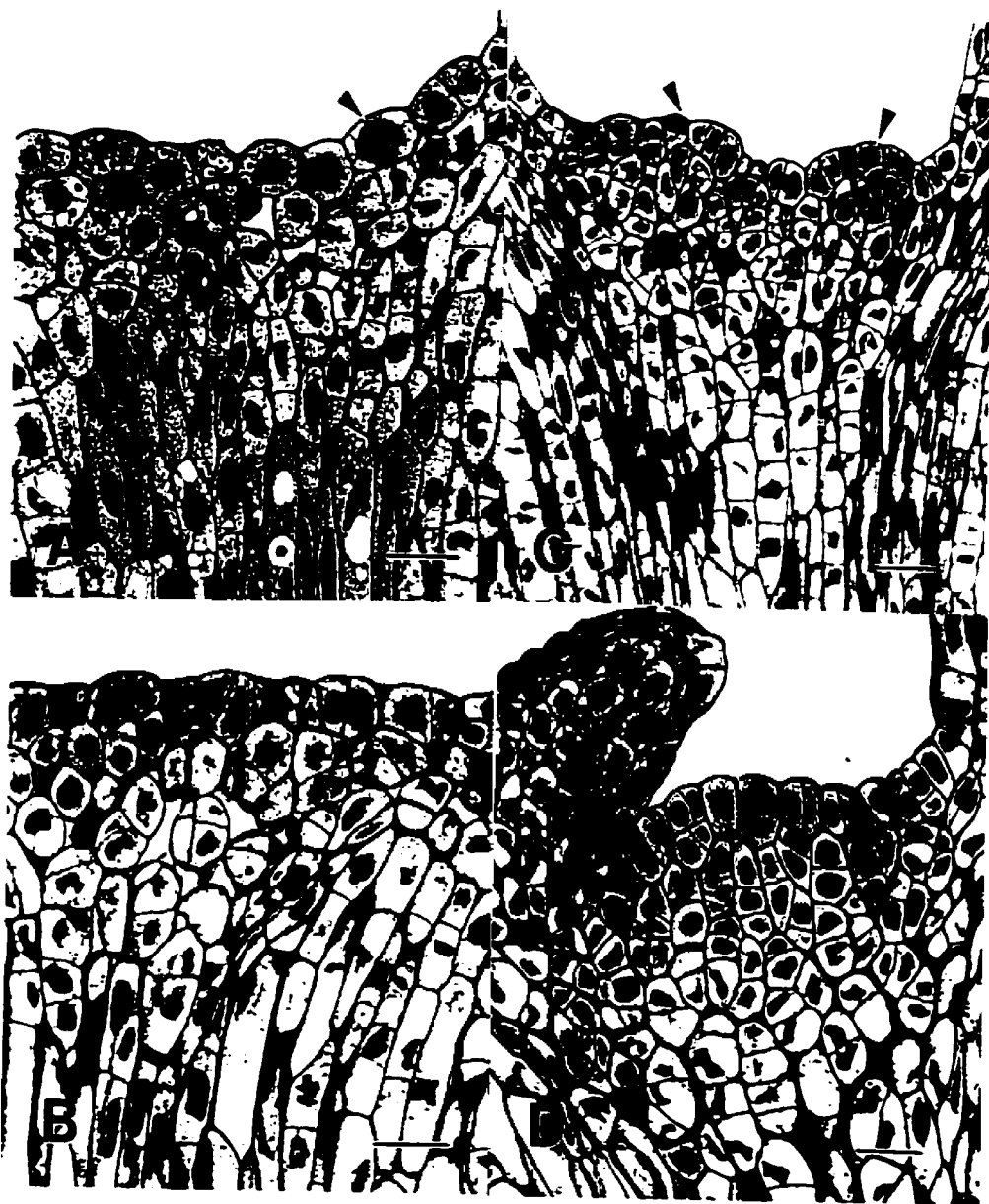
Fig. 3.5. Morphology of a "well organized" shoot apical meristem of a germinating control embryo of the (E)WS1 line. After 2 days of germination. many meristematic cells started to accumulate phenolic compounds (arrowheads) (A). Many of these meristems were not able to develop further. Increased accumulation of phenolic substances (arrowheads) (B) and appearance of large vacuoles (*) in the sub-apical cells resulted in meristem abortion (C). In some instances, cessation of meristem growth was a later event, as poorly developed shoot primordia were observed (D). All scale bars = 40 μm.
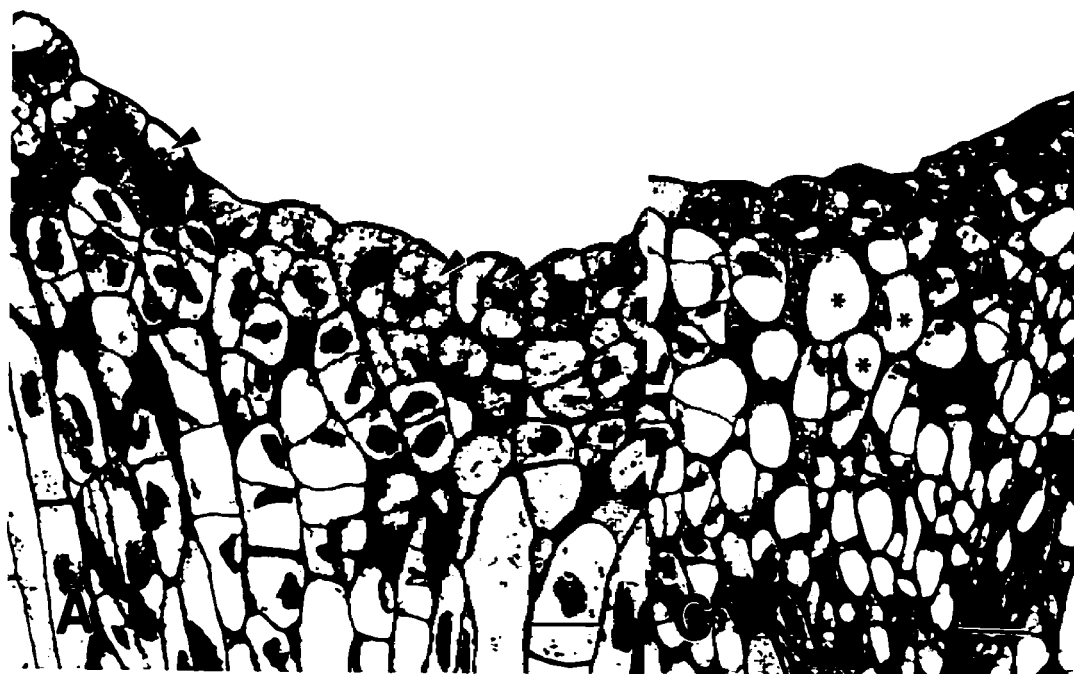
Fig. 3.6. Morphology of a poorly organized shoot apical meristem of a germinating control embryo of the (E)WS1 line. Accumulation of phenolics (arrowhead) in disorganized meristems started 4 days after germination (**A**). Many of these meristems continued to accumulate phenolics (arrowheads) within the cells of the apical and sub-apical layers (**B**). With time. more cells of the shoot pole showed deposition of phenolics (arrowheads). Cell division was seldom observed in these meristems which were not able to undergo any further development (**C**). All scale bars = 40 µm.
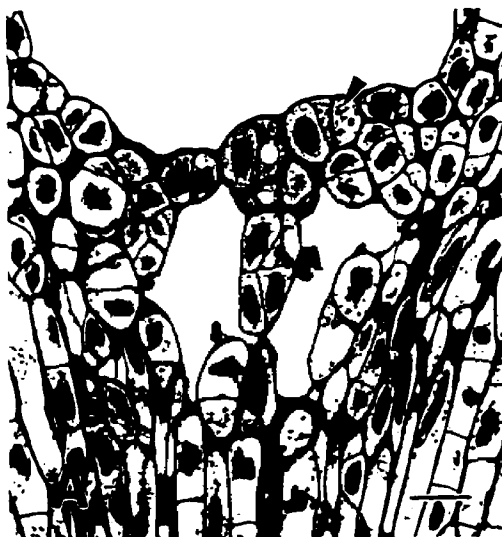
Fig. 3.7. Colour micrographs of a well organized meristem (**A**) and a poorly organized meristem disrupted by the presence of intercellular air spaces (*) (**B**). Accumulation of phenolic compounds (arrowheads), which stained blue with toluidine blue O stain, was observed within the apical and sub-apical cells of both meristems after a few days of germination. All scale bars = 40 μm.
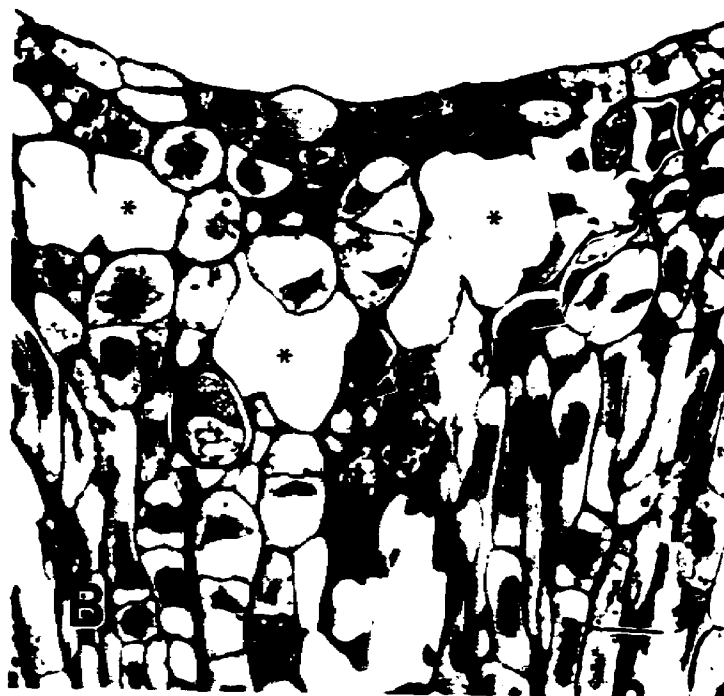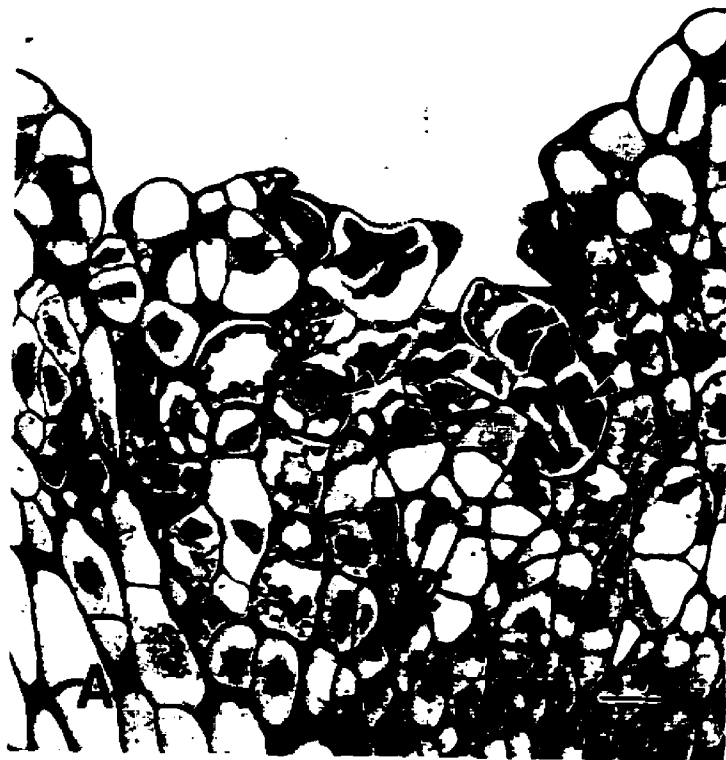
Fig. 3.8. Morphology of a poorly organized shoot apical meristem of an AA-treated embryo of the (W)WS1 line. Although disrupted by the presence of many intercellular air spaces, some meristematic cells of AA-treated embryos were able to divide after 2 days of germination (arrowheads) **(A)**. Continual mitotic activity resulted in the formation of meristematic primordia (arrowhead) **(B)**.

Accumulation of phenolic substances was seldom observed in these meristems, which continued to increase in size **(C)**. The appearance of well developed leaf primordia, observed at day 14, indicated that these shoot primordia were able to behave as functional meristems **(D)**. All scale bars = 40 μm.

Fig. 3.9. Morphology of a poorly organized shoot apical meristem of an AA-treated embryo of the (E)WS1 line. In some instances, applications of AA stimulated mitotic divisions at the base of the cotyledons near the apical meristem (arrowheads) **(A)**. Continuation of cell proliferation resulted in the formation of meristematic zones (arrowhead) **(B)**, which in some cases covered a large portion of the cotyledons (arrowheads) **(C)**. More than one shoot primordium could develop from the meristematic zones. These structures were capable of independent growth and behaved as functional meristems (arrowheads) from which leaf primordia originated **(D)**. All scale bars = 40 μm.
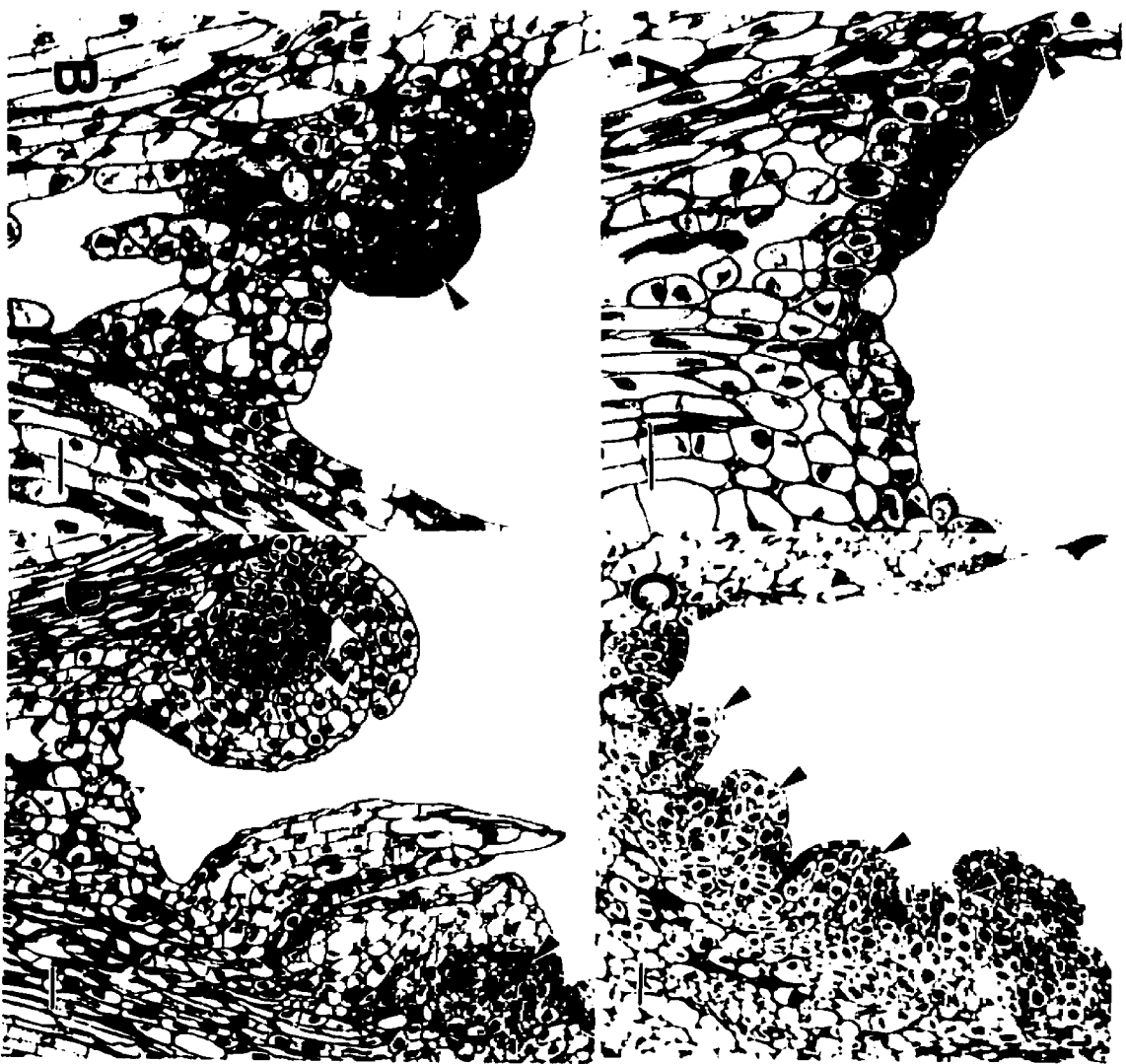
lycorine, an inhibitor of the AA biosynthetic pathway, resulted in a profound inhibition of cell division. This effect was however reversed after the concentration of AA in the tissue was increased (Liso et al., 1984). Thus, it appears that, like other systems, AA plays a fundamental role during the germination of white spruce somatic embryos.

Another interesting point emerging from this study is the profound beneficial effect of AA on shoot conversion, which increased from 47% (control) to 79% (100 μM AA) (Table 3.1). This finding indicates that AA treatments may be required for the induction/maintenance of mitotic activity in the shoot apical meristems of germinating embryos. As observed in Figures 3.7 and 3.8, in fact, even poorly organized shoot apical meristems, which under normal circumstances would not be able to reactivate at germination, are induced to resume growth and to form new leaf primordia if treated with AA. The promotive effect on shoot conversion, not observed for the root (Table 3.1), may be ascribed to either a differential distribution of the AA level along the embryo axis, with a higher concentration at the shoot pole, or a difference in sensitivity of the two apical meristems to AA. Although often observed in root systems (Liso et al., 1984; 1988; Cordoba-Pedregosa et al., 1996), the promotive effect of AA on cell division has also been reported on shoots. As observed by Joy et al. (1988), treatments with AA increased the shoot-forming capacity of tobacco callus.

Despite the positive correlation between cellular AA level and cell division, very few studies deal with the molecular mechanisms controlling this effect. As shown in Figure 3.5, the shoot apices of control embryos that fail to convert at germination are characterized by heavy accumulation of phenolics. Shoot apical meristems of AA-treated embryos, on the other hand, do not seem to accumulate phenolic compounds (Fig. 3.8). Although without any direct evidence, it appears that if deposited in large amount, phenolic substances can compromise the growth and conversion of the embryos at germination. As reported by Fry (1986), both cell proliferation and elongation in plant cells are strictly dependent upon the mechanical properties of the wall architecture. Both processes, in fact, are inhibited by those mechanisms which induce cell wall stiffening, including the peroxidase-driven polymerization of phenolics in the apoplast. In pine, for

example, high levels of cell-wall bound peroxidase activity and increasing polymerization of phenolic substances have been associated with growth cessation of hypocotyl segments (Sanchez et al., 1995). Thus, it is possible that in white spruce. AA may control the amount of phenolics accumulated in the meristems through the regulation of cellular peroxidases. Several studies showing the inhibitory effect of AA on major secretory peroxidases both in vivo and in vitro support this notion. As documented by De Gara and Tommasi (1999), in fact, applications of AA may facilitate the removal of hydrogen peroxide, the substrate of the secretory peroxidases, through the activation of ascorbate peroxidase (see Fig. 1.3, Chapter 1). Along the same line, Cordoba-Pedregosa et al. (1996) observed a positive correlation between onion root growth and the AA-inhibition of cell-wall bound guaiacol and ferulic acid peroxidases. Similarly, in vitro studies also indicate that AA inhibits peroxidase activity (Takahama and Oniki. 1992: 1994). The effect of AA on major cellular peroxidases during white spruce somatic embryo germination will be discussed later (Chapter 4).

In conclusion, data from this study clearly indicate that exogenous applications of AA may be beneficial for the conversion of white spruce somatic embryos. possibly by regulating the production/deposition and cross-linking of phenolic compounds in meristematic cells. This study also provides evidence that. besides improving the quality of the embryos at maturation (Kong and Yeung, 1995), the "rescue" of meristems at germination represents an alternative strategy for improving the post-embryonic performance of the embryos.

CHAPTER FOUR

The effects of ascorbic acid on the activities of major peroxidases of germinating white spruce somatic embryos

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Introduction

As reported in Chapter 3. the post-embryonic performance of white spruce somatic embryos is dependent upon the reactivation of the apical meristems during the initial phases of germination. In the embryos of the (E)WS1 line. unable to undergo successful conversion, cell division in the apical meristems was either never observed or was initiated but not continued. Structural studies also indicated that heavy accumulation of phenolic substances in the meristematic cells preceded meristem abortion and cessation of plantlet development. Applications of ascorbic acid (AA) were found to enhance shoot meristem conversion. as well as to reduce the accumulation of phenolic compounds (see Chapter 3). It is generally accepted that deposition and cross-linking of phenolic compounds. driven by peroxidases. affect the architecture of the cell wall and ultimately cell elongation and cell division. As discussed by Fry (1986). increased peroxidase activity and cross-linking of phenolic compounds often result in the stiffening of the cell wall and in the reduction of both cell elongation and cell division.

Thus. in order to comprehend the regulation of cell-wall extensibility during plant growth and development, including germination, focus must be placed on the biological control of peroxidase activities. This includes the synthesis and secretion of peroxidases, as well as the supply of substrates, such as phenolic compounds and hydrogen peroxide (see Biggs and Fry. 1987). Besides their involvement in the cross-linking of phenolic components. peroxidases have been involved in the formation of other bonds. including isodityrosine bonds between glycoproteins. such as extensin. and diferulate bridges between polysaccharides polymers (Biggs and Fry, 1987). Activities of peroxidases are usually low in meristematic tissues, characterized by rapid growth. and high in differentiated tissues, where both cell division and elongation have ceased to occur (Fry. 1986; De Gara and Tommasi, 1999).

Since previous studies have demonstrated the strong inhibitory effect of AA on peroxidase activities both in vitro (Takahama and Oniki. 1992; 1994) and in vivo (Cordoba-Pedregosa et al., 1996), it is suggested that exogenous applications of AA may enhance shoot meristem conversion of white spruce somatic embryos by reducing the activity of cellular peroxidases. Such a reduction in peroxidase activity would increase cell wall extensibility, thus allowing cell elongation and division in the apical poles of the germination embryos. In order to test this hypothesis, the activities of major cellular peroxidases. including ferulic acid and guaiacol peroxidases, were measured after altering the endogenous AA content in germinating white spruce somatic embryos. This was achieved by using L-galactono-γ-lactone (GL), the last precursor of the de novo biosynthesis of AA. and lycorine (L). an alkaloid extracted from various members of Amaryllidaceae (Evidente et al.. 1983), which inhibits the last reaction leading to the synthesis of AA (see Fig. 1.2. Chapter 1).

Materials and Methods

Plant material

Establishment and maintenance of white spruce cell cultures

Establishment and maintenance of embryogenic tissue of white spruce was described in Chapter 2. This experiment, however, was carried out with the embryos of the (E)WSC line, as the (E)WS1 line, utilized in Chapters 2 and 3, lost its embryo-forming ability. Compositions of both induction and maintenance media were the same as those reported in Chapter 2.

Somatic embryo maturation, partial drying treatment, and germination

Methods for the induction of somatic embryo development. partial drying, and

promotion of germination were similar to those described in Chapter 2.

Light microscopy studies

These were carried out as described in Chapter 2.

Alterations of the endogenous AA levels and tissue sampling

Several agents were utilized in this experiment for altering the endogenous AA level of the germinating embryos. Ten microliters of water (control), 1 mM ascorbic acid (AA), 2 mM L-galactono-γlactone (GL), and / or 100 μM lycorine (L), were applied every 24 hours directly on germinating embryos. Each solution, pH 5.8, was filter sterilized with a 0.2 μm Nalgene syringe filter. Prior to each application, the embryos were transferred onto a fresh germination medium to avoid accumulation of degradation products of the agents applied the previous day. Embryos were collected at days 0, 2, 6, and 12, rinsed in distilled water, and utilized for AA measurements and enzyme assays.

Lycorine was a gift received from Dr. Laura De Gara (Universita' di Bari, Italy).

AA measurements

AA measurements were carried out as described in Chapter 2.

Enzyme extraction and assays of peroxidase activities

Germinating embryos were homogenized at 4° C with a medium containing 50 mM potassium phosphate buffer, pH 6.0, 1 mM EDTA, 2 mM DTT, Triton X-100 (0.5% v/v), and PVPP (1.5% w/v). After centrifugation at 4° C for 20 min at 16.000 g, the supernatant was desalted on a pre-packed column of Sephadex G-25 (NAP-25, Pharmacia, Biotech Inc, Baie d'Urfe, ON, Canada) and utilized for the enzyme assays.

Ascorbate peroxidase (AAP) activity was measured as described in Chapter 2.

For AAP, 1 unit of activity was defined as 1 nmol AA oxidized min^{-1} mg $protein^{-1}$

Guaiacol peroxidase (GP) activity was determined as described by

Cordoba-Pedregosa et al. (1996). The reaction, carried out in a mixture containing 50

mM sodium phosphate, pH 6.0, 0.06% H_2O_2, and 3 mM guaiacol, was followed

spectrophotometrically for 1 min at 470 nm.

Ferulic acid peroxidase (FP) activity was measured in a reaction mixture similar

to that utilized for GP determination, without guaiacol and with the addition of 0.2 mM

ferulic acid. The reaction was followed for 1 min at 310 nm (Cordoba-Pedregosa et al.,

1996). The final volumes for both guaiacol and ferulic acid peroxidases were 1 ml. For

both guaiacol and ferulic acid peroxidases, 1 unit of activity was defined as 0.1 Abs. min^{-1}

mg $protein^{-1}$.

Protein was determined according to Lowry (1951) using BSA as a standard.

Effects of AA on the activity of peroxidases in vitro

In order to determine whether applications of AA affected the activity of

peroxidases in vitro, 20 µg of protein extracted from 6-d germinating embryos was

utilized for assaying guaiacol and ferulic acid dependent peroxidases as previously

described. In these experiments, however, increasing concentrations of AA (25, 50, and

100 µM) or 2 units of ascorbate oxidase (Sigma), were added prior to the reading.

Statistical analysis

This was conducted as described in Chapter 2.

Native PAGE analysis of total peroxidase activity

Control and AA-treated embryos were homogenized in the same extraction buffer

utilized for enzyme assay (1:2 w/v). After centrifugation at 4°C for 20 min at 16000 g, the supernatant was used for electrophoresis analysis. Native-PAGE was performed using a stacking gel containing 4.3% acrylamide (acrylamide/N'N'-bis-methylene-acrylamide 35.5:1 mixture) and a running gel containing 7.3% acrylamide with a running buffer composed of 4 mM Tris-HCl. pH 8.3. and 38 mM glycine. After the run at 4° C. the gel was washed with distilled water. For staining. the gels were incubated at 4° C for 30 min in 0.05 M sodium phosphate buffer pH 6.0. containing 0.6% hydrogen peroxide. 0.1 M $CaCl_2$, and 3 mM 3-amino-9-ethyl carbazole (AEC). a non-specific electron donor for total peroxidases (Soltis et al..1983). Peroxidase isoenzymes were located as red bands on a transparent background as a result of the oxidation of AEC. Control experiments were performed in the absence of hydrogen peroxide. For all experiments. 150 μg of protein were loaded in each lane.

Histochemical localization of total peroxidase activity in germinating embryos

Germinating embryos were fixed in freshly prepared 4% paraformaldehyde buffered with 0.05 M phosphate buffer. pH 6.9. Fixation was carried out at 4° C on a rotator (Pelco. Redding. CA. USA) for 3 hours. After washing. the tissue was dehydrated in a graded ethanol series (changes were carried out every 30 min) and then infiltrated and embedded in Historesin (Leica. Markham. ON. Canada) (Yeung and Law 1987). In order to avoid the loss of enzymatic activity. the entire procedure (fixation. dehydration. infiltration. and embedding) was completed in 24 h. Sectioning was carried out with glass knives on a Reichert-Jung 2040 Autocut microtome. Serial longitudinal sections were cut at a thickness of 4 μm. For histochemical localization of total peroxidase activity. the sections were stained for 15 min at room temperature in 50 mM sodium phosphate buffer. pH 6.0. containing 0.02% hydrogen peroxide and 3 mM 3.3-diaminobenzidine (Hall and Sexton. 1972). Control slides were incubated in a similar medium devoid of hydrogen peroxide. The preparations were examined and photographed with a Leitz Aristoplan light microscope.

Results

Effect of culture conditions on conversion frequency

Similar percentages of conversion were observed in the somatic embryos of the (E)WSC line, germinated in the presence of water (control), ascorbic acid (AA), and L-galactono-γ-lactone (GL). Applications of lycorine alone (L) or L-galactono-γ-lactone plus lycorine (GL+L), resulted in a statistically significant decrease of conversion frequency (21% and 27% respectively), whereas only a slight decline in the percentage of embryos able to convert into viable plantlets was observed in the presence of ascorbic acid plus lycorine (AA-L) (Table 4.1).

Endogenous AA content of treated embryos

At day 2, all embryos, irrespective of treatment, had similar endogenous AA levels (Fig. 4.1). Differences in the AA content among the treatments were however observed during the following days. Applications of lycorine (L) and L-galactono-γ-lactone plus lycorine (GL+L) resulted in a decline of the endogenous AA content. After 12 days of germination, the ascorbate levels of (L)- and (GL+L)-treated embryos were 7.7 nmol embryo^{-1} and 6.6 nmol embryo^{-1} respectively, compared to 15.3 nmol embryo^{-i} of the control counterparts. Higher levels of endogenous ascorbate were measured in embryos germinated in the presence of exogenous ascorbate (AA), L-galactono-glactone (GL), and ascorbate plus lycorine (AA+L) at any stage of germination. As revealed by statistical analyses, significant differences were observed in the endogenous AA level between control embryos and all other treatments after day 2.

Effect of culture conditions on the structure of the apical meristems

After 2 days of germination, the shoot poles of the control embryos had a flat-

shaped appearance. At this stage, no mitotic activity was detected within the apical meristem (Fig. 4.2 A). Resumption of mitotic activity in the meristematic cells during the following days of germination resulted in successful meristem conversion, as denoted by the appearance of a functional dome-shaped meristem (Fig. 4.2 B). A similar growth of the shoot poles was also observed in AA-treated embryos and in those AA+L-treated embryos which were able to convert into viable plantlets. Although mitotic activity was observed in the shoot apices of L-treated embryos (Fig. 4.2 C), accumulation of phenolic substances and vacuolation of the meristematic cells resulted in growth cessation and meristem abortion (Fig. 4.2.D).

The root apical meristems of the somatic embryos were composed of large, cytoplasmic cells. In all treatments, cell division was first detected at day 2, especially in the procambial region (Fig. 4.2 E). Mitotic activity was never observed in the cells of the root apical meristems of L-treated embryos. These cells started to elongate, and they were characterized by the presence of large vacuoles. After 6 days in culture, the formation of intercellular air spaces resulted in cell separation and meristem abortion (Fig. 4.2 F). In the other treatments (C, AA, and AA+L), reactivation of the root meristems, as denoted by the presence of mitotic figures, was observed after day 4 (Fig. 4.2 G).

Effect of culture conditions on peroxidase activity

Effects of AA on guaiacol, ferulic acid, and ascorbic acid peroxidase activity

Germination of white spruce somatic embryos in the presence of ascorbic acid (AA), L-galactono-glactone (GL), and ascorbate plus lycorine (AA+L), resulted in a general decrease in activity of both ferulic acid- and guaiacol-dependent peroxidases, compared to the control (Figs. 4.3 and 4.4).

The most pronounced decrease in the activities of these enzymes was observed at day 2, when the activities of ferulic acid and guaiacol peroxidases in (AA)-, (GL)-, and (AA+L)-treated embryos were less than half those measured in their control (C)

Table 4.1. Effects of exogenously applied ascorbic acid (AA), L-galactono-γ-lactone (GL), and lycorine (L) on conversion (root elongation and emergence of new leaf primordia) of white spruce somatic embryos. Values are expressed as means of three independent experiments ± SE.

Treatment	% of embryo conversion
Control	88.5±9.9
AA	85.4±5.6
AA+L	61.8±6.7
L	21.1±2.3
GL	77.9±11.5
GL+L	26.9±4.8

Fig. 4.1. Effects of ascorbic acid (AA). L-galactono-γ-lactone (GL), and lycorine

(L) treatments on the endogenous AA concentration of germination white

spruce somatic embryos. Values are means ± SE. C, control.
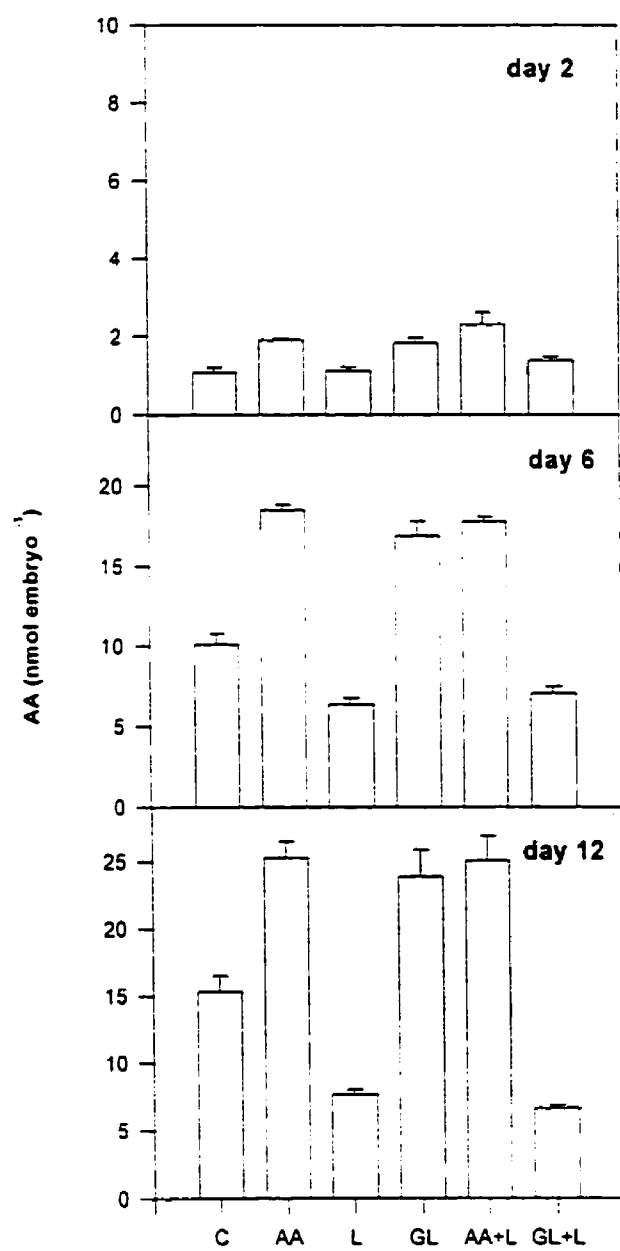
counterparts (Fig. 4.3 and 4.4). These differences were statistically significant. Exogenous applications of lycorine (L) resulted in high peroxidase activity after 2 days in germination. whereas slight, but not significant differences in activities were observed between control (C) embryos and (GL+L)-treated embryos at any stage of germination (Figs. 4.3 and 4.4). At day 2, the activity of ascorbate peroxidase (AAP) was lower in L- and GL+L-treated embryos compared to that observed in their control counterparts. The activity of this enzyme was similar in all treatments during the later stages of embryo germination (Fig. 4.5).

Histochemical localization of peroxidases in shoot and root apices

Histochemical localization of peroxidase activity was determined in shoot and root apices of control. AA-. L-, and AA+L-treated embryos. Staining for total peroxidase activity. not observed in partially dried embryos (data not shown), was first detected after 2 days of germination. At this stage, both shoot and root meristems of (L)-treated embryos stained more intensely for peroxidase activity than control embryos (Fig. 4.6 A-D). In contrast, exogenous applications of ascorbate (AA) and ascorbate plus lycorine (AA+L) decreased the staining intensity during the first two days of germination (Fig. 4.6 E-H). Upon further development. however. with the emergence of root and new leaf primordia. a similar staining pattern was observed between these treatments (AA and AA+L) and control embryos in both apical meristems (data not shown).

Electrophoretic profile of peroxidases

As shown by native PAGE analysis (Fig. 4.7). total peroxidase activity only appeared after 2 days of germination. At this stage, four bands were detected in control embryos whereas only two in AA-treated embryos. The other two complementary bands appeared in AA-treated embryos at day 4. and they were less intensely stained than those observed in their control counterparts. A similar staining profile between AA-treated and control embryos was observed after 8 days of germination (Fig. 4.7).

Fig. 4.2. Micrographs showing the effects of the endogenous AA levels on the structure of the apical meristems of germinating embryos. After 2 days the shoot apical meristem of control embryos maintained a flat-to-concave appearance (arrowhead) (**A**). Resumption of mitotic activity during the following days resulted in the growth of the meristem, as denoted by the appearance of a dome-shaped shoot primordium (arrowhead) after 8 days in the germination medium (**B**). Although cell divisions occurred in the shoot pole of L-treated embryos (arrow), accumulation of phenolics was initiated in many meristematic cells (arrowheads) (**C**). In these meristems, continual deposition of phenolics (arrowheads) and vacuolization of the sub-apical cells (*) resulted in growth cessation (**D**). In the root pole of control embryos, cell divisions were first observed at day 2 (arrows). At this stage, however, no mitotic activity was detected in the cells of the root apical meristem (arrowheads) (**E**). Cells within the root meristems of L-treated embryos never divided. Cell elongation, vacuolation (*), and formation of intercellular air spaces (arrowhead) resulted in meristem abortion (**F**). Reactivation of the cells of the root apical meristems of control embryos, as denoted by the presence of mitotic figures (arrowheads), occurred after 4 days of germination (**G**). All scale bars = 40 μm. A similar growth pattern of the shoot and root apical meristems was observed for control and AA- and AA+L-treated embryos.

Fig. 4.3. Effects of ascorbic acid (AA), L-galactono-γ-lactone (GL), and lycorine (L) applications on the activity of ferulic acid-dependent peroxidase of germinating white spruce somatic embryos. Values are means ± SE. C, control. 1 unit = 0.1 Abs. min^{-1} mg protein^{-1}.
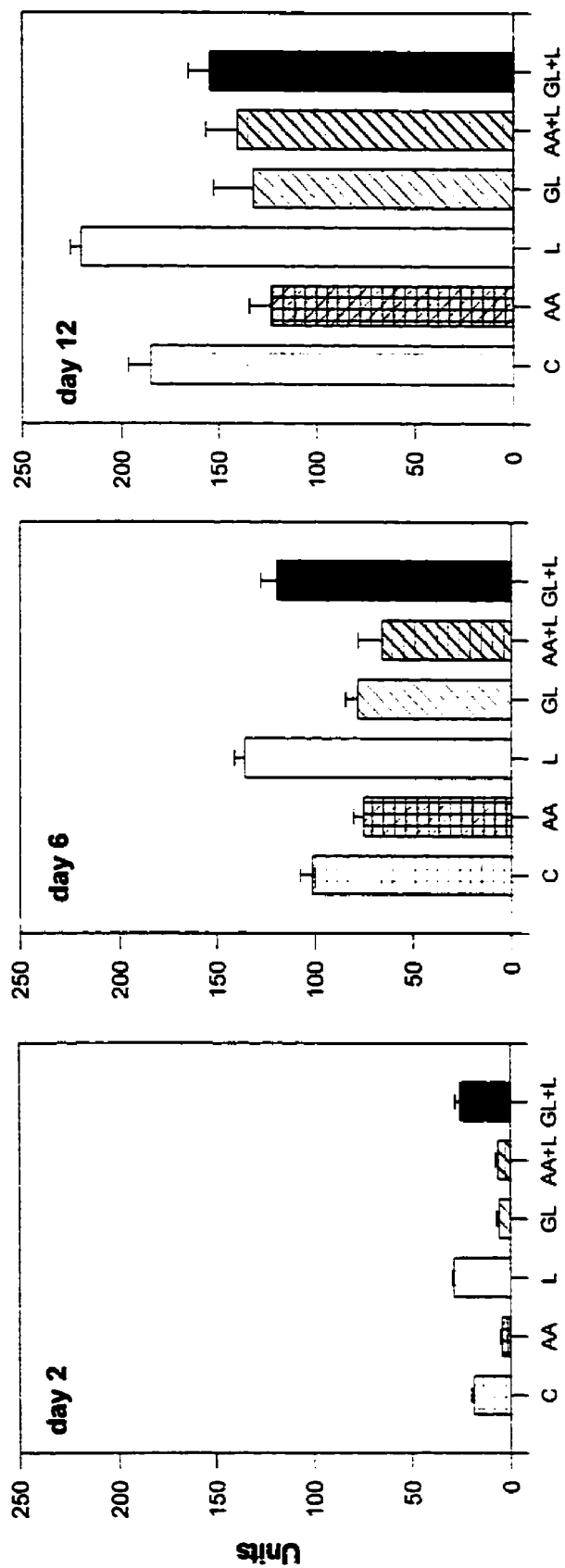
Fig. 4.4 Effects of ascorbic acid (AA), L-galactono-γ-lactono (GL), and lycorine (L) applications on the activity of guaiacol-dependent peroxidase of germinating white spruce somatic embryos. Values are means ± SE. C, control. 1 unit = 0.1 Abs. min^{-1} mg $protein^{-1}$.
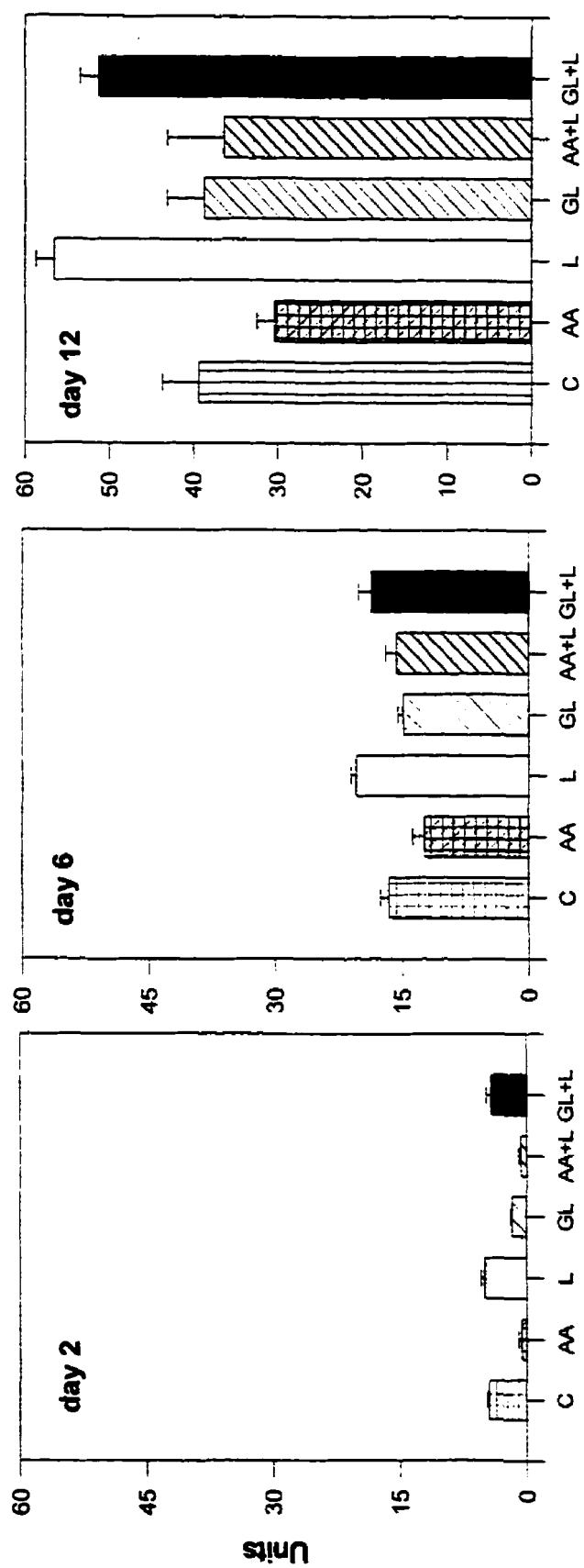
Fig. 4.5. Effects of ascorbic acid (AA), L-galactono-γ-lactone (GL), and lycorine (L), applications on the activity of ascorbic acid-dependent peroxidase of germinating white spruce somatic embryos. Values are means ± SE. C, control. 1 unit = 1 nmol AA oxidized min^{-1} mg $protein^{-1}$.
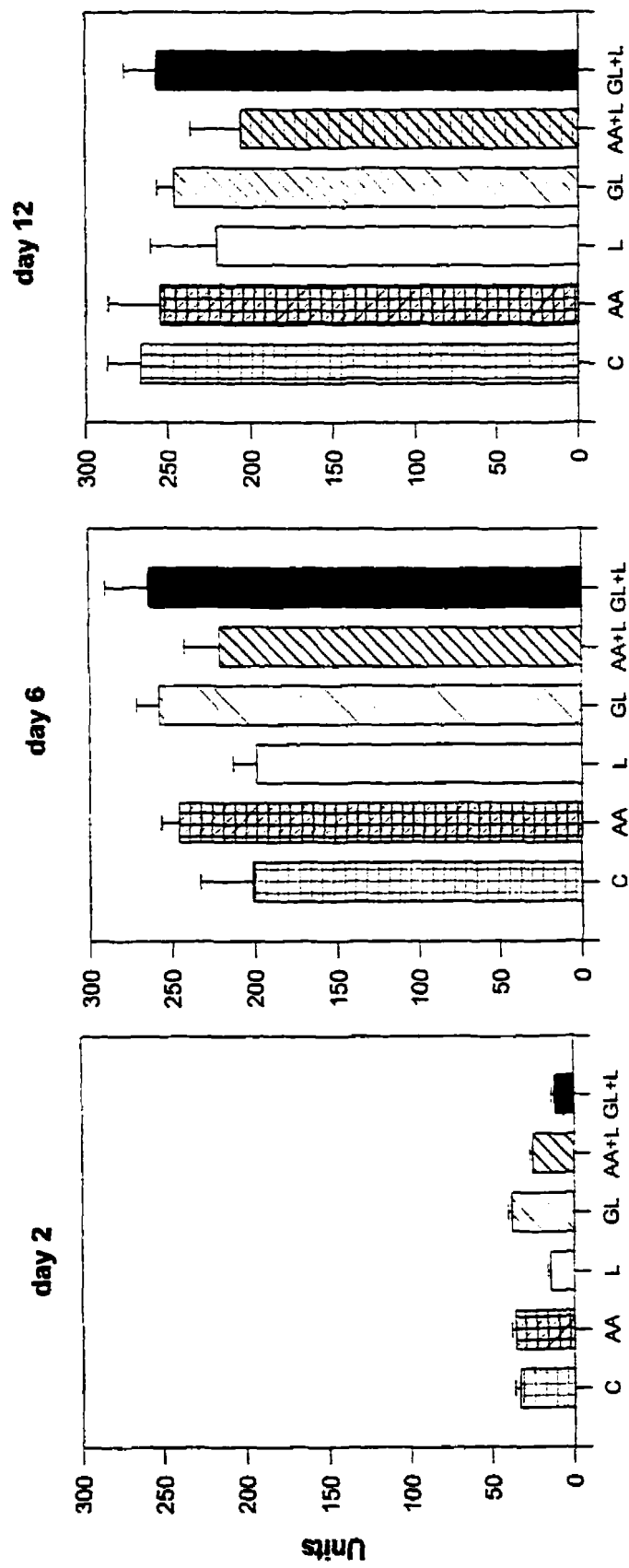
Fig. 4.6. Histochemical localization of peroxidase activity in shoot and root apices of control and L-, AA-, and AA+L-treated embryos after 2 days of germination. In the root of control embryos peroxidase activity was mainly localized in the cortical cells (arrows). Low peroxidase activity was detected in the cells of the root apical meristem (arrowheads) **(A)**. Reduced staining for peroxidases was observed in the cells of the shoot apical meristem (arrowhead) of control embryos **(B)**. In L-treated embryos. intense staining for peroxidases was detected along the entire root apex. including the root meristem (arrowheads) **(C)**. Many cells of the shoot apical meristem (arrowhead) of L-treated embryos stained for peroxidases. Peroxidase activity was detected in both cell wall and cytoplasm of these cells **(D)**. A general reduction in staining intensity was observed in the root apex of AA-treated embryos, especially in the cells of the root meristem (arrowheads) **(E)**. Little staining was observed in the cells of the shoot apical meristem (arrowhead) of AA-treated embryos **(F)**. Compared to that observed in the control embryos. a reduction in staining intensity was observed in the cells of the root apical meristem (arrowheads) **(G)** and shoot apical meristem (arrowhead) **(H)** of AA+L treated embryos. Slides were incubated in the presence of 3-amino-9-ethyl carbazole. a non-specific electron donor for total peroxidases (see Materials and Methods). All scale bars = 40 μm.

A   B

C   D

E   F

G   H
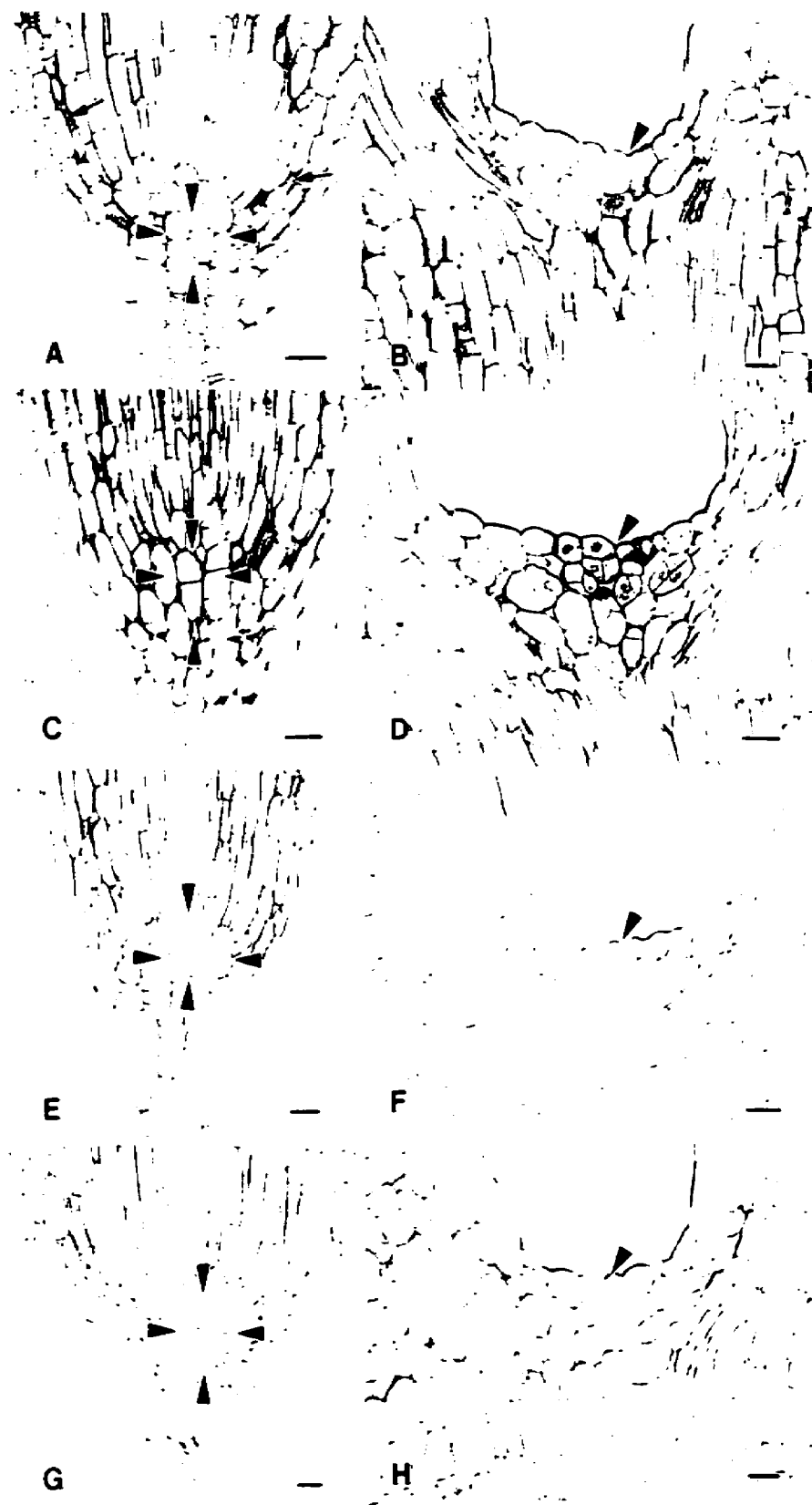
Effects of AA on peroxidase activity in vitro

Both ferulic acid and guaiacol dependent peroxidases were inhibited in the presence of exogenous applications of ascorbate (AA) (Fig. 4.8). The inhibition time of both peroxidases was proportional to the AA concentrations, as the AA inhibition ceased as soon as all AA in the reaction mixture was oxidized. Oxidation of the endogenous AA from the extraction buffer by applications of ascorbate oxidase (AAO) did not affect the rates of ferulic acid and guaiacol oxidation (Fig. 4.8).

Discussion

Reactivation of the apical meristems at germination is a complex process involving cell division and differentiation. Through a series of coordinated cell divisions, both shoot and root apical meristems contribute cells to the surrounding tissues, thus allowing the growth of the embryo. In white spruce somatic embryos, such a process is fundamental for successful conversion into viable plantlets, as failure to resume mitotic activity in meristematic regions results in poor post-embryonic performance of the embryos (Kong and Yeung, 1992). Ascorbic acid (AA) is a common compound in plants that might be physiologically involved in the regulation of cell division and differentiation. As reported in Chapter 3, exogenous applications of AA have been found to enhance the conversion frequency of white spruce somatic embryos, possibly by promoting cell division in structurally disorganized meristems. A similar promotive effect of AA on cell division has also been documented in several other systems (Liso et al., 1984, 1988; Arrigoni et al., 1997; de Pinto et al., 1999). Despite these observations, however, very few hypotheses have been proposed dealing with possible mechanisms of action of AA during the process. Besides reducing the cell cycle time by promoting the progression of cells from G1 to S (Liso, 1988), Takahama and Oniki (1994) have suggested the possibility that AA might regulate cell growth through the inhibition of peroxidase-dependent polymerization of phenolics and cross-linkage of cell-wall components.

Fig. 4.7. Electrophoretic profiles of proteins extracted from germinating embryos and stained for total peroxidase activity. AA. ascorbic acid: C. control. 150 mg protein were loaded per lane. Arrows indicated major bands.
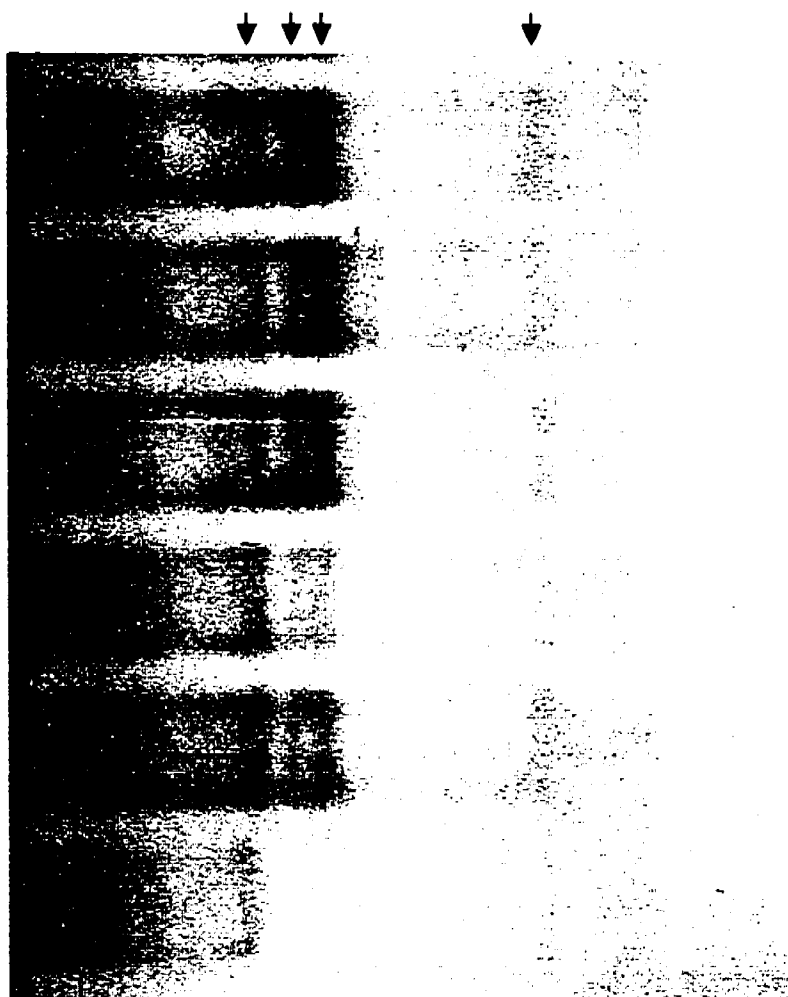
Fig. 4.8. In vitro effect of ascorbic acid (AA) and ascorbic acid oxidase (AAO) applications on ferulic acid and guaiacol dependent peroxidases. Increasing concentrations of AA (25. 50. and 100 μM) or 2 units of AAO were added to the reaction mixture prior to reading. For each experiment 20 μg of protein extract were utilized.

Guaiacol oxidation

Ferulic acid oxidation

control
AA (100 μM)
AA (50 μM)
AA (25 μM)
AAO (2u)
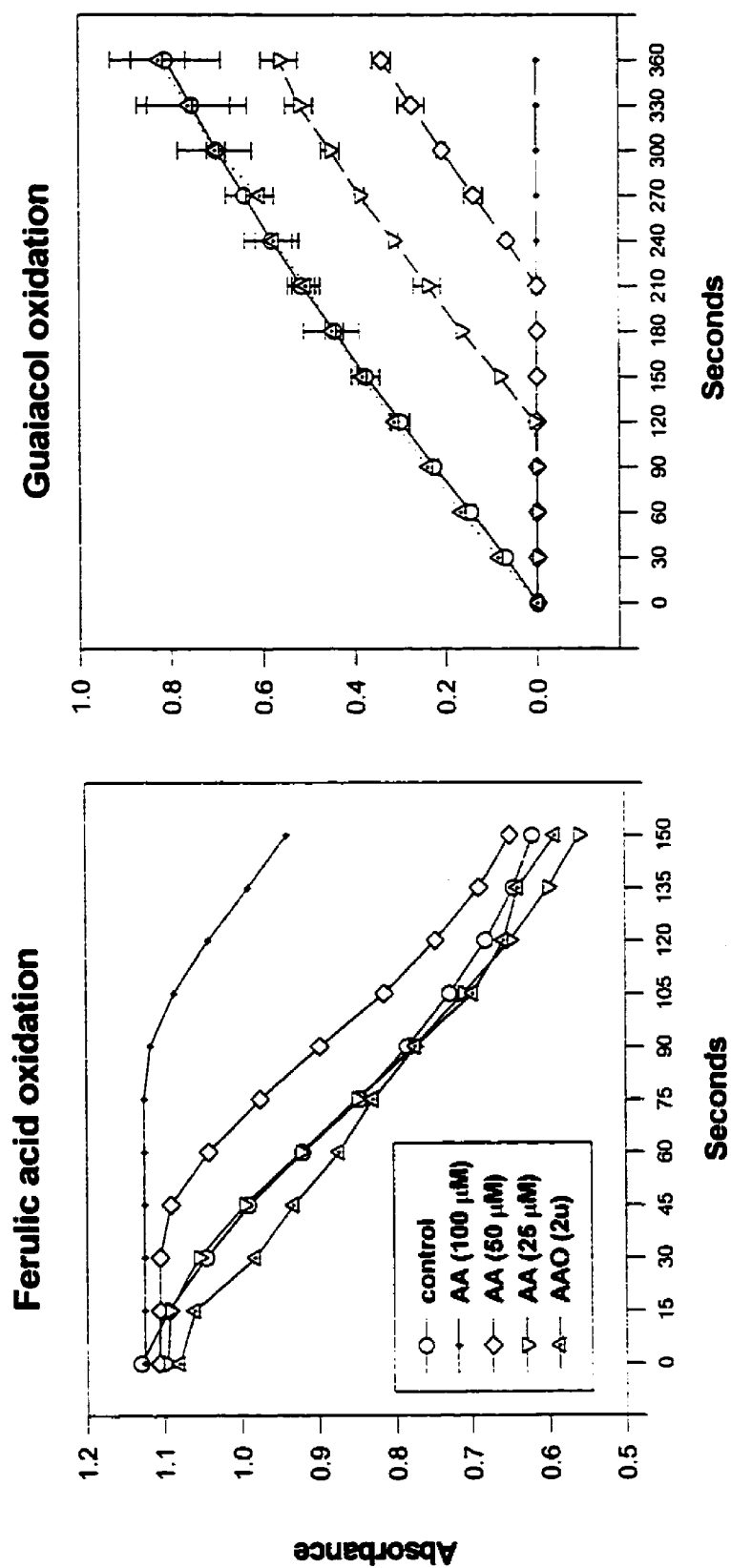
In plants the last step in the AA biosynthetic pathway is catalyzed by a dehydrogenase using GL as a substrate (Isherwood et al., 1954). GL is promptly converted to AA in the cell and lycorine (L) prevents GL conversion to AA. by inhibiting the activity of L-galactono-1,4-lactone dehydrogenase (De Gara et al., 1994) (Fig. 1.2, Chapter 1). Such control of the de novo pathway of AA also occurs in white spruce somatic embryos as applications of GL and L have opposite effects on the endogenous AA level (Fig. 4.1). The result of the present study also indicates that treatments that lower the endogenous AA levels. i.e. L and GL+L (Fig. 4.1), reduce the ability of white spruce somatic embryos to convert into viable plantlets (Table 4.1). Conversely, an increment in the endogenous AA levels caused by applications of AA, AA+L, and GL (Fig. 4.1) did not affect the conversion frequency of the embryos (Table 4.1). This finding is different from that reported in Chapter 3, where exogenous applications of AA enhanced the post-embryonic performance of the embryos of the (E)WS1 line. This difference in result is most likely due to the fact that the endogenous AA level is higher in the (E)WSC embryos, utilized in this study, compared to their (E)WS1 counterparts. As such, it is suggested that exogenous applications of AA do not have any effect on systems characterized by high levels of endogenous AA.

The different response in conversion frequency observed in white spruce somatic embryos following alterations of the endogenous AA might be partially ascribed to changes in activities of major cellular peroxidases. In vivo studies on peroxidase activities clearly indicate that a decrease in endogenous AA concentration by applications of L and GL+L correlates with a general increase in guaiacol and ferulic acid oxidation (Figs. 4.3 and 4.4). This trend was also confirmed by histochemical studies, as both shoot and root meristems of L-treated embryos. which failed to convert at germination. stained more intensely for total peroxidase activity than their control counterparts (Fig. 4.6 A-D). Conversely, treatments that increase the endogenous level of AA. i.e. AA. AA+L. and GL. inhibited both ferulic acid and guaiacol-dependent peroxidases, especially during the first days of embryo germination (Figs. 4.3 and 4.4). Similarly, AA- and AA+L- treated embryos showed reduced staining of peroxidases in both shoot and root poles (Fig. 4.6 E-H). Thus a negative correlation between peroxidase activity against guaiacol and ferulic

acid and successful conversion of the embryos exists in white spruce. A similar correlation was also observed in other systems, including gymnosperms (Goldberg et al. 1987; Valero et al., 1991). Sanchez et al. (1995) suggested that accumulation and dimerization of ferulic acid, catalyzed by peroxidases, cause cell wall stiffening and growth cessation in pine tissue. Inhibition of growth in white spruce may be controlled by similar events, as besides increasing peroxidase activity, applications of lycorine resulted in phenolic accumulation in the shoot apical meristems (Fig. 4.2C,D).

The key role played by AA in the regulation of peroxidase dependent polymerization of phenolic compounds has been emphasized by many studies. De Gara and Tommasi (1999) proposed that AA might facilitate the removal of H_2O_2 by acting as a substrate for ascorbate peroxidase (AAP). Thus, increasing activity of AAP would reduce the availability of hydrogen peroxide utilized by secretory peroxidases for cell wall cross-linking. Besides its higher affinity for H_2O_2 (De Gara and Tommasi, 1999), a negative correlation between AAP activity and major peroxidases activities was observed in several systems, including white spruce zygotic embryogenesis (Stasolla and Yeung, unpublished observation). In the present study, however, no correlation was observed between the patterns of guaiacol and ferulic acid oxidation (Figs. 4.3 and 4.4) and AAP activity, which was similar for all the different treatments after day 2 (Fig. 4.5). Increasing amount of AA in germinating white spruce somatic embryos might affect peroxidases directly, possibly by altering their synthesis and/or secretion. As shown by the electrophoretic studies, in fact, AA seems to change the peroxidase isoenzyme profile, especially during the initial stages of germination (Fig. 4.7). Similar changes in peroxidase activity were also reported by Cordoba-Pedregosa et al. (1996), who found a negative correlation between peroxidase activity and growth of onion root incubated with AA.

Finally, endogenous AA might be utilized as an electron donor not only by ascorbic-specific peroxidases, such as AAP, but also by other classes of secretory peroxidases (Welinder and Gajhede, 1993; Mehlhorn et al., 1996). The in vitro studies conducted in this experiment confirm this observation. Peroxidase activities measured with ferulic acid and guaiacol were completely abolished, but released after the AA that was present in the assay medium was oxidized (Fig. 4.8). Takahama and Oniki (1992,

1994) obtained similar results for apoplastic and cell-wall bound coniferyl alcohol-dependent peroxidases and demonstrated the inhibitory effect of AA in the reaction.

Although without direct evidence. in white spruce cells, alterations of symplastic levels of AA may result in changes of AA levels in the apoplast. In this compartment. AA might affect cell wall properties by regulating peroxidase activity. Although the apoplastic AA level was not measured in this experiment. Rautenkranz et al. (1994) showed that symplastic AA may be transported to the apoplast. Transport of AA from the cytoplasm to the apoplast has also been observed by Castillo and Greppin (1988) in leaves of *Sedum album* and by Luwe et al. (1993) in spinach leaves subjected to ozone exposure.

In conclusion. the results of this investigation clearly indicate that the endogenous AA level plays a fundamental role during white spruce somatic embryogenesis. With the resumption of mitotic activity in the apical meristems. a large availability of AA may be needed for inhibiting the activity of major peroxidases. including those involved in wall cross-linking. This would result in cell wall relaxation which is necessary to allow cell division in meristematic cells. Therefore. manipulations of the culture medium which increase the endogenous AA content would be beneficial for the post-embryonic growth of the somatic embryos. especially for those lines characterized by low AA content. It must be mentioned. however. that although important for the resumption of mitotic activity in white spruce somatic embryos, relaxation of cell wall components alone cannot account for the AA enhancement of embryo conversion. Other cellular processes more directly involved with nucleic acid synthesis and cell division must be regulated by AA for successful conversion to occur.

CHAPTER FIVE

The effects of exogenous ascorbic acid applications on purine and pyrimidine nucleotide biosynthesis of germinating white spruce somatic embryos

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Introduction

As shown in Chapter 3, exogenous applications of AA have been found to increase the percentage of germination and conversion of white spruce somatic embryos of the (E)WS1 cell line by inducing organized cell division in morphologically disrupted meristems. The participation of AA in several cellular processes, including cell division, has been emphasized by many investigations. De Gara et al. (1996) reported that in pea stem the content of AA is high in the meristematic region and gradually declines in more differentiated tissues. Similarly, high levels of AA were observed during the initial stages of angiosperm (Arrigoni et al., 1992) and gymnosperm (Chapter 2) seed development, characterized by rapid cell divisions, and at the inception of germination of both zygotic (De Gara et al., 1997) and somatic (Chapter 2) embryos. Furthermore, an increase in the endogenous AA content has been found to promote cell proliferation in several systems, such as *Allium cepa* root, where AA induces a progression of meristematic cells from G1 to S (Liso et al., 1988), tobacco cultured cells (de Pinto et al., 1999), *Zea mays* root (Kerk and Feldmann, 1995), and cambial cells of *Lupinus albus* roots (Arrigoni et al., 1997). Applications of ascorbic acid were also found to enhance tobacco shoot organogenesis (Joy IV et al., 1988). Conversely, when AA content is experimentally lowered by lycorine, cells which are normally competent to divide, arrest in the G1 phase of the cell cycle (Liso et al., 1984). It is surprising, however, that despite the tight correlation between AA and plant growth emerging from these data, there is little information

concerning the inductive mechanisms of AA on cell proliferation (De Tullio et al., 1999).

Purine and pyrimidine nucleotides are essential components of nucleic acid synthesis, as well as cellular intermediates participating in bio-energetic processes and in several metabolic cycles (Ross, 1981). Synthesis of nucleotides can occur through a de novo pathway or through a salvage pathway, which utilizes preformed bases and nucleosides. Independent degradation pathways for both purine and pyrimidine nucleotides also exist in plant cells (Figs. 5.1 and 5.2).

Alterations in the pattern of purine and pyrimidine metabolism have often been associated with physiological changes during plant growth both in vivo (Nygaard, 1973) and in vitro (Hirose and Ashihara, 1984; Stasolla et al., 2001c). Dramatic changes in purine and pyrimidine nucleotide biosynthesis were also reported in germinating seeds (Price and Murray, 1969; Guranowski and Barankiewicz, 1979; Nobusawa and Ashihara, 1983). These studies have mainly revealed the importance of the salvage pathway during the initial stages of germination, when a large amount of nucleotides is needed in support of the resumption of mitotic activity, prior to the restoration of the de novo biosynthetic pathway.

On the basis of the above, the main objective of this study was to determine whether applications of AA enhanced the conversion frequency of white spruce somatic embryos by altering the biosynthesis of purine and pyrimidine nucleotides. Studies on purine metabolism were carried out by incubating the somatic embryos with ^{14}C-labeled adenine and adenosine, intermediates of the salvage pathway, and inosine, a substrate of the degradation pathway (Fig. 5.1). The de novo pathway was not investigated due to the high cost of the precursor phosphoribosylaminoimidazole carboxamide (PRAICA). For pyrimidines, ^{14}C-labeled orotic acid, uridine, and uracil were utilized as markers for the de novo, salvage, and degradation pathways respectively (Fig. 5.2). Furthermore, the activities of the key enzymes involved in purine and pyrimidine metabolism were measured.

Materials and Methods

Plant material

Generation of embryogenic tissue

This was carried out as described in Chapter 3

Promotion of embryo development and partial drying treatment (PDT)

Promotion of somatic embryo development and imposition of the partial drying treatment was performed as described in Chapter 3

Embryo germination and AA applications

Germination of somatic embryos and AA applications were carried out as outline in Chapter 3.

Chemicals

[8-^{14}C]adenine (1.96 TBq mol^{-1}), [8-^{14}C]adenosine (1.67 TBq mol^{-1}), [8-^{14}C]inosine (2.07 TBq mol^{-1}), [6-^{14}C]orotic acid (1.85 TBq mol^{-1}), [2-^{14}C]uridine (2.0 TBq mol^{-1}), and [2-^{14}C]uracil (1.85 TBq mol^{-1}) were purchased from Moravek Biochemicals Inc (Brea, CA, USA).

Metabolism of ^{14}C-labeled purine and pyrimidine precursors

Metabolism of ^{14}C-labeled purine precursors was carried out as described by Ashihara et al. (2000, 2001) and Stasolla et al. (2001c). Partially dried embryos (10 days

PDT) and germinating embryos (about 40) were incubated in 2 ml of liquid germination medium in a 25-ml Erlenmeyer flask with a central well. A strip of filter paper. impregnated with 100 μl of 20% KOH. was placed in a glass tube in the well. The flasks were then incubated at 25° C for 2 hours and 18 hours on gentle agitation. At the end of the incubation period the moistened filter paper was collected and placed in 10 mL of distilled water in a 50 ml-Erlenmeyer flask. After 24 hours. 0.5 ml of distilled water was utilized for measurement of radioactivity (CO_2 fraction). In the meantime. the embryos were washed in distilled water, frozen in liquid nitrogen, and homogenized in a ice-cold mortar with 3 ml of 6% perchloric acid (PCA). After centrifugation at 8,000 g for 10 min. the supernatant (PCA-soluble fraction) was collected. neutralized with KOH, and evaporated to dryness below 40° C. The powder was then resuspended in 50% ethanol and loaded onto a microcrystalline cellulose plate (200 mm x 200 mm. Whatman K2. 150 micron; VWR-Canlab. Edmonton. Canada) for fractionation. A solution of butanol-acetic acid-water (BAW) 4:1:2 (v/v) was used as the solvent. The plates. developed for 24 hours by using an imaging plate BAS III (Fuji Photo Film Co.. ltd.. Tokyo. Japan). were analyzed with a Bio-Imaging analyzer (Type FLA-2000. Fuji Photo Film Co.. Ltd. Tokyo. Japan). The pellet was re-suspended in an ethanol-ether (1:1. v/v) mixture at 50° C for 20 min. After centrifugation at 8,000 g for 10 min the supernatant (lipid fraction) was removed and utilized for measurement of radioactivity. The remaining pellet was first boiled in 6% PCA for 15 minutes, and then washed in 6% PCA. The combined supernatant constituted the nucleic acid fraction (DNA+RNA). After neutralization with KOH. the nucleotide fraction was evaporated to dryness at 40° C and fractionated by thin layer chromatography in BAW 4:1:2 (v/v). Analysis of the plates was carried out as previously described. Cold and hot standards of bases and nucleosides were also loaded onto the plates for the recognition of the bands obtained.

Enzyme assays

Determination of the specific activities of the enzymes (see Figs. 5.1 and 5.2) were carried out as reported by Ashihara et al. (2000. 2001) and Stasolla et al. (2001c).

Somatic embryos (400-500 mg fresh weight) were homogenized in 50 mM HEPES-NaOH buffer (pH 7.6), 2 mM NaEDTA, 2 mM dithiothreitol (DTT), and 0.5% sodium ascorbate. The slurry was centrifuged at 20,000 g for 20 min at 4° C, and the supernatant was subsequently desalted on a pre-packed column of Sephadex G-25 (NAP-25, Pharmacia, Biotech Inc., Baie d'Urfe, ON, Canada). For each enzyme assay 35 μL of the desalted extract was incubated for 2, 5, and 10 min at 30° C in the following reaction mixtures (total volume 100 μL).

1) Adenine, uracil, and uridine phosphoribosyltransferase. These were determined in a reaction mixture containing 30 mM HEPES-NaOH buffer (pH. 7.6), 10 mM MgCl$_2$, 1 mM DTT, 0.6 mM 5-phosphoribosyl-1-pyrophosphate (PRPP), and 45 μM of labeled substrate ([8-^{14}C]adenine, [2-^{14}C]uracil, or [6-^{14}C]orotic acid).

2) Adenosine, inosine, and uridine kinases. These were determined in a reaction mixture containing 30 mM HEPES-NaOH buffer (pH. 7.6), 10 mM MgCl$_2$, 1 mM DTT, 3.75 mM ATP, and 45 μM of labeled substrate ([8-^{14}C]adenosine, [8-^{14}C]inosine, or [2-^{14}C]uridine).

3) Nucleoside phosphotransferase (NPT). The reaction mixture was similar to that utilized for kinases, except that ATP was replaced by 3.75 mM AMP.

4) Adenosine nucleosidase (ARN). The reaction mixture was similar to that used for adenosine kinase, but ATP was omitted from the mixture.

5) Phosphoribosylpyrophosphate synthetase (PRPP synthetase). This was determined in a reaction mixture containing 30 mM HEPES-NaOH buffer (pH 7.6), 10 mM MgCl$_2$, 1 mM DTT, 3.75 mM ATP, 3.75 mM ribose-5-phosphate, and 46 μM [6-^{14}C]orotic acid.

Final specific activities of [8-^{14}C]adenine, [8-^{14}C]adenosine, [8-^{14}C]inosine, [2-^{14}C]uracil, [2-^{14}C]uridine, and [6-^{14}C]orotic acid were 0.36, 0.30, 0.38, 0.34, 0.37, and

0.38 MBq mmol^{-1} respectively. The reactions were terminated by the addition of 10 mL 70% PCA. After neutralization with KOH, each reaction mixture was further evaporated to dryness, and the pellet was resuspended in 55 mL of 50% ethanol. Each sample (7 µl) was loaded onto the TLC plate and developed in BAW 4:2:1 (v/v). For adenosine nucleosidase. distilled water was utilized as a solvent (Poulton and Butt. 1976). Detection and measurement of the substrate and product obtained in each reaction was performed by using imaging plates and Bio-Imaging analyzer. as described above. Cold and hot standards were also loaded onto the plates for the identification of the bands.

Three independent experiments were performed for all the enzyme assays. The specific activity of the enzymes was expressed as pkat mg^{-1} protein.

Results

Purine metabolism

Total uptake of purine intermediates

As shown in Table 5.1. on an embryo basis, the uptake of [8-^{14}C]adenine. [8-^{14}C]adenosine. and [8-^{14}C]inosine was extremely low at the end of the partial drying period (10d PDT) and sharply increased after transfer to the germination medium. Comparisons between control and AA-treated embryos revealed that the total uptake of adenine and adenosine was lower in the former throughout the germination period. This difference was more pronounced at day 2. when the rates of uptake of AA-treated embryos were almost double those observed in the control counterparts.

Fig. 5.1. Possible metabolic fate of exogenously supplied [8-^{14}C]adenosine. [8-^{14}C]adenine and [8-^{14}C]inosine during the germination of white spruce somatic embryos. Enzymes: (1) AK. adenosine kinase and NPT (adenosine), nucleoside phosphotransferase (2) APRT, adenine phosphoribosyltransferase; (3) ARN, adenosine nucleosidase; (4) IK. inosine kinase and NPT (inosine), nucleoside phosphotransferase (5) inosine-guanosine nucleosidase ; (6) and (7) xanthine dehydrogenase; (8) uricase; (9) allantoinase. Enzymes measured are represented by numbers in brackets [ ].

Glutamine

[3]

Adenosine $\longrightarrow$ Adenine

De novo pathway

[1] [2]

ATP $\rightleftarrows$ $\rightleftarrows$ AMP $\longrightarrow$ IMP $\rightarrow$ $\rightarrow$ GMP $\rightleftarrows$ $\rightleftarrows$ GTP

[4]

Salvage pathway

Nucleic acids   Inosine   Nucleic acids

5

Hypoxanthine

6

Xanthine

Degradation pathway

7

Uric acid

8

Allantoin

9

Allantoic acid

$CO_2 + NH_3$
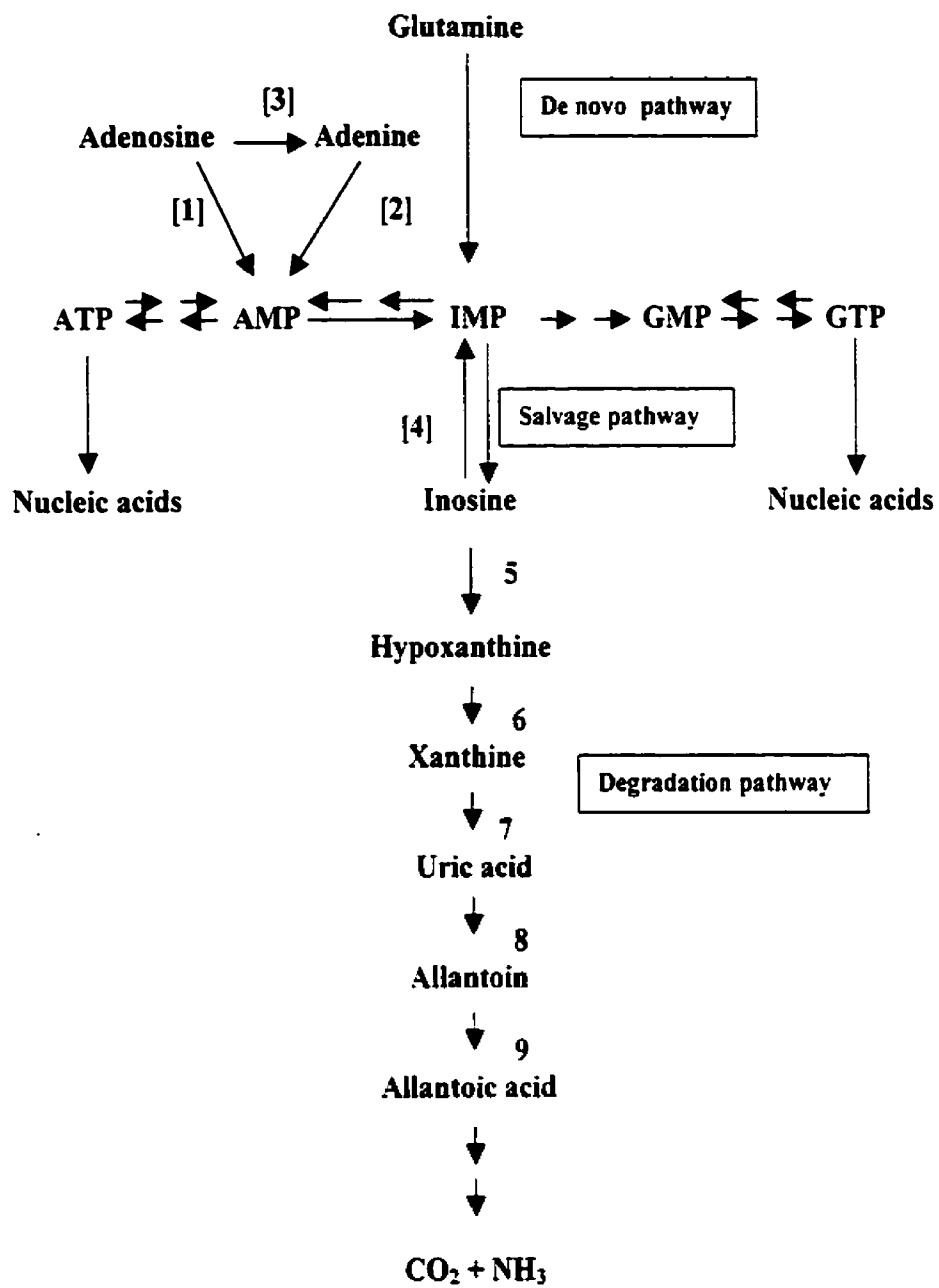
Fig. 5.2. Possible metabolic fate of exogenously supplied [2-^{14}C]uracil, [2-^{14}C]uridine, and [6-^{14}C]orotic acid during germination of white spruce somatic embryos. R-5-P. ribose-5'-phosphate: PRPP. 5'-phosphoribosylpyrophosphate: OMP, orotidine monophosphate: UMP. uridine monophosphate: UDP. uridine diphosphate: UTP. uridine triphosphate: CTP. cytidine triphosphate. Enzymes involved are: (1) PRPP synthetase. (2) OPRT, orotate phosphoribosyltransferase: (3) UK, uridine kinase and NPT (uridine), non specific phosphotransferase: (4) UPRT, uracil phosphoribosyltransferase; (5) uridine nucleosidase; (6) uridine phosphorylase; (7) uracil reductase and/or dihydrouracil dehydrogenase; (8) dihydropyriminase; (9) orotidine-5'-monophosphate decarboxylase; (10) nucleoside monophosphate kinase; (11) nucleoside diphosphate kinase. Enzymes measured are represented by numbers in brackets [ ].
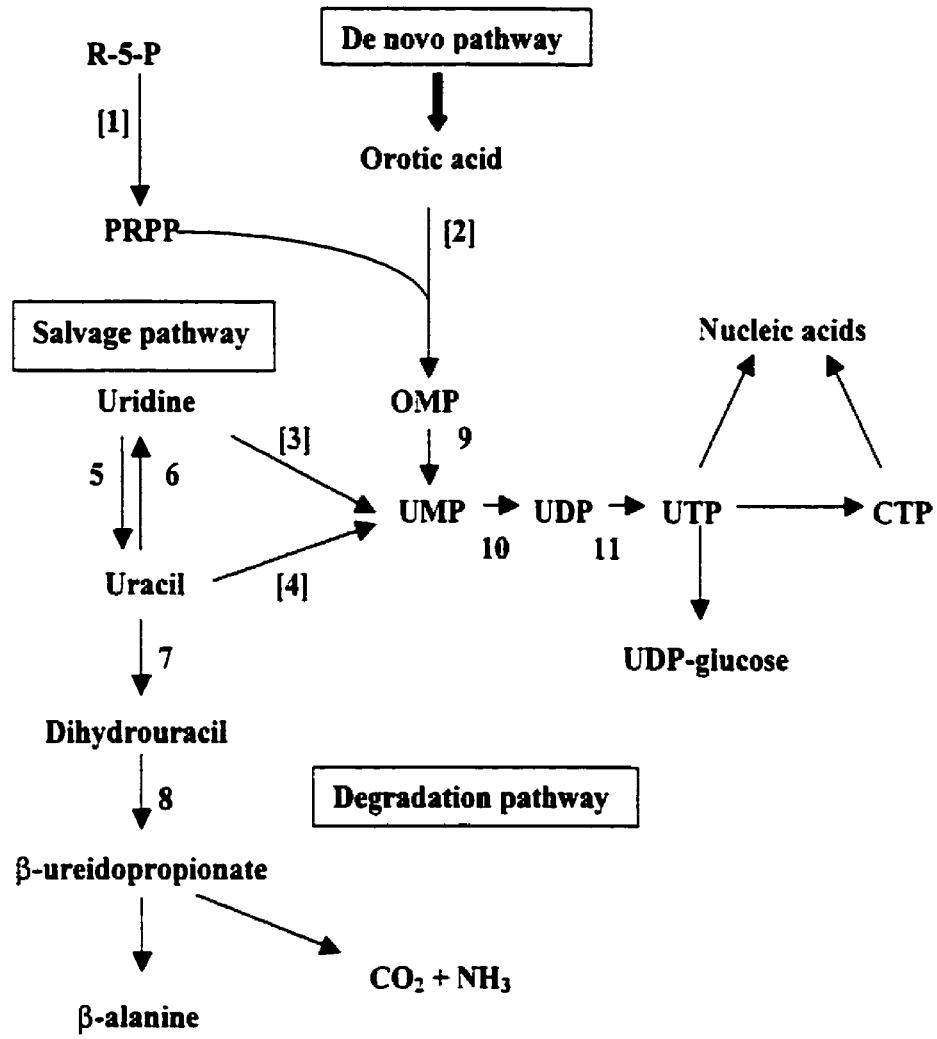
Table 5.1. Uptake of ^{14}C-labeled purine precursors by white spruce somatic embryos germinated in the absence (control) or in the presence (AA) of 10^{-4}M ascorbic acid. Somatic embryos were incubated for 2 hours in the presence of the labeled precursors. Values are expressed as Bq embryo^{-1}±SE. PDT, partial drying treatment; germ., germination.

^{14}C-precursor	10 d PDT	2 d germ.		4 d germ.		6 d germ.	
		Control	AA	Control	AA	Control	AA
[2-^{14}C]Adenine	25.1±3.8	121.3±17.4	248.1±2.3	368.4±20.4	370.2±16.0	542.3±21.6	675.4±45.1
[2-^{14}C]Adenosine	11.1±1.0	69.2±2.9	143.5±4.7	176.0±11.0	224.5±33.5	363.1±21.6	452.2±25.0
[8-^{14}C]Inosine	7.3±0.7	44.7±1.8	80.9±10.8	220.4±20.4	195.1±4.9	258.8±10.4	262.7±0.8

Purine salvage pathway: metabolism of [8-^{14} C]adenine and adenosine

The purine salvage pathway of germinating white spruce somatic embryos was investigated by following the metabolic fate of [8-^{14}C]adenine and [8-^{14}C]adenosine. For adenine, the distribution of radioactivity among the various metabolites after a 2-h incubation is shown in Fig. 5.3. At the end of the drying period (10 days PDT) almost all the radioactivity taken up by the embryos was equally distributed among nucleotides, mainly ATP+ADP. and unmetabolized adenine. During the first 4 days of germination a large fraction of ^{14}C in both control and AA-treated embryos was recovered into salvage products, i.e. nucleic acids and nucleotides. Among the nucleotides, the ATP+ADP fraction was the most labeled. Only a small amount of [8-^{14}C]adenine was catabolized as ureides and CO_2. Differences in adenine metabolism between control and AA-embryos were observed between day 4 and day 6 after germination.

In control embryos, the amount of radioactivity recovered as ATP+ADP remained almost constant, whereas that recovered into the ureide fraction sharply increased from 7 pmol embryo^{-1} at day 4 to almost 90 pmol embryo^{-1} at day 6. Conversely, in AA-treated embryos the proportion of radioactivity recovered as nucleotides, mainly ATP+ADP. almost doubled from day 4 to day 6. whereas that recovered as ureides only increased slightly (Fig. 5.3).

The profile of adenosine metabolism closely resembled that of adenine. As germination progressed, a pronounced increase of [8-C^{14}]adenosine was utilized for nucleotide (mainly ADP+ATP) and nucleic acid (Fig. 5.4) biosynthesis. As with adenine, differences between control and AA-treated embryos appeared between day 4 and day 6. In control embryos, the ADP+ATP fraction remained constant, whereas an increasing amount of adenosine was degraded as ureides (4.7 pmol embryo^{-1} at day 4 and 64 pmol embryo^{-1} at day 6). An opposite trend was observed in AA-treated embryos where the radioactivity from the ATP+ADP fraction sharply increased, whereas a much smaller increase in the amount of radioactivity recovered into the ureide fraction was observed at day 4 and day 6 (Fig. 5.4).

Purine degradation pathway: metabolism of [8-¹⁴C]inosine

The purine degradation pathway was investigated by following the metabolic fate of ¹⁴C-labeled inosine. At the beginning of the germination period (10 days PDT) the small amount of radioactivity taken up by the embryos was almost equally distributed among ureides and unmetabolized inosine. As germination progressed, a gradual increase in radioactivity recovered as degradation products, i.e. ureides and CO_2 was observed in both control and AA-treated embryos. After 6 days of germination the amount of inosine catabolized as ureides and CO_2 was 23 pmol embryo^{-1} and 71 pmol embryo^{-1} in control embryos, and 18 pmol embryo^{-1} and 75 pmol embryo^{-1} in AA-treated embryos (Fig. 5.5)

Enzymes of purine metabolism

Changes in activity of the enzymes participating in the recycling of purine bases and nucleosides are shown in Fig. 5.6. Among the enzymes involved in the salvage of adenine and adenosine, both adenine phosphoribosyltransferase (APRT) and adenosine kinase (AK) markedly increased as germination progressed. The activity of the former was higher than that of the latter throughout the length of the experiment. When compared with those of control embryos, the specific activities of APRT and AK were higher in AA-treated embryos at day 6. Very low activity of nucleoside phosphotransferase NPT (adenosine) was observed in both control and AA-treated embryos throughout the germination period. Adenosine nucleosidase (ARN), the enzyme responsible for the conversion of adenosine to adenine, was not detected at the end of the drying period and after 2 days in germination. A limited presence of this enzyme was however noticed as germination progressed (days 4 and 6). Among the enzymes responsible for the salvage of inosine, inosine kinase (IK) was absent in dried embryos, but it gradually increased during germination. Low activity of inosine phosphotransferase [NPT (inosine)] was detected throughout the germination period.

Fig. 5.3. Distribution of radioactivity into different labeled fractions of germinating white spruce somatic embryos incubated for 2 hours with [2-^{14}C]adenine (A). Embryos were germinated in the absence (open bars) or in the presence (solid bars) of 10^{-4}M ascorbic acid. Values are expressed as means ± SE. (x0.5), values were multiplied by a factor of 0.5. (x10), values were multiplied by a factor of 10. PDT, partial drying treatment; germ., germination.

Adenine

(I) Nucleic acids

(II) ATP+ADP (x0.5)

(III) AMP (x10)

(IV) Ureides

(V) CO$_2$

(VI) A

pmoles / embryo

10d PDT   2d germ.   4d germ.   6d germ.

Fig. 5.4. Distribution of radioactivity into different labeled fractions of germinating white spruce somatic embryos incubated for 2 hours with [2-^{14}C]adenosine (AR). Embryos were germinated in the absence (open bars) or in the presence (solid bars) of 10^{-4}M ascorbic acid. Values are expressed as means ± SE. (x0.5), values were multiplied by a factor of 0.5; (x10), values were multiplied by a factor of 10.

Adenosine
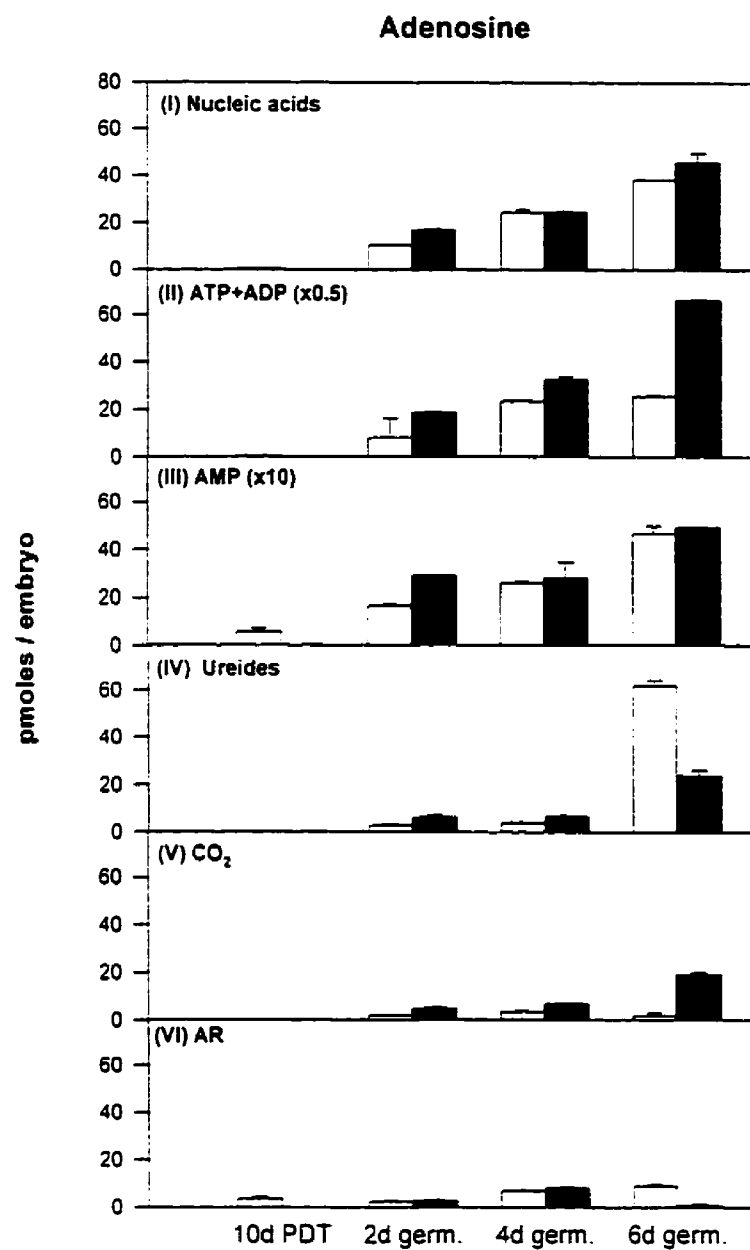
Fig. 5.5. Distribution of radioactivity into different labeled fractions of germinating white spruce somatic embryos incubated for 2 hours with [8-^{14}C]inosine (IR). Embryos were germinated in the absence (open bars) or in the presence (solid bars) of 10^{-4}M ascorbic acid. Values are expressed as means ± SE. (x10), values were multiplied by a factor of 10. PDT, partial drying treatment; germ., germination.

Inosine

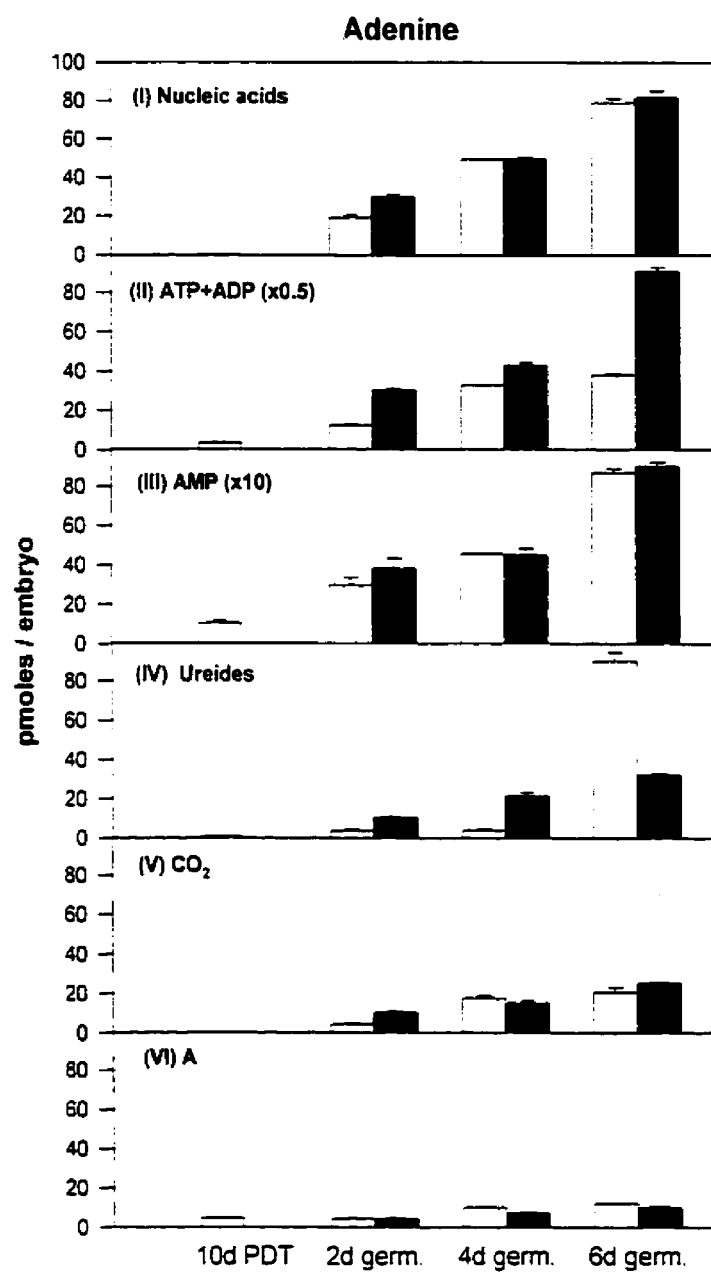
pmoles / embryo

(I) Nucleic acids (x10)

(II) ATP+ADP (x10)

(III) AMP (x10)

(IV) Ureides

(V) CO₂

(VI) IR (x10)

10d PDT   2d germ.   4d germ.   6d germ.
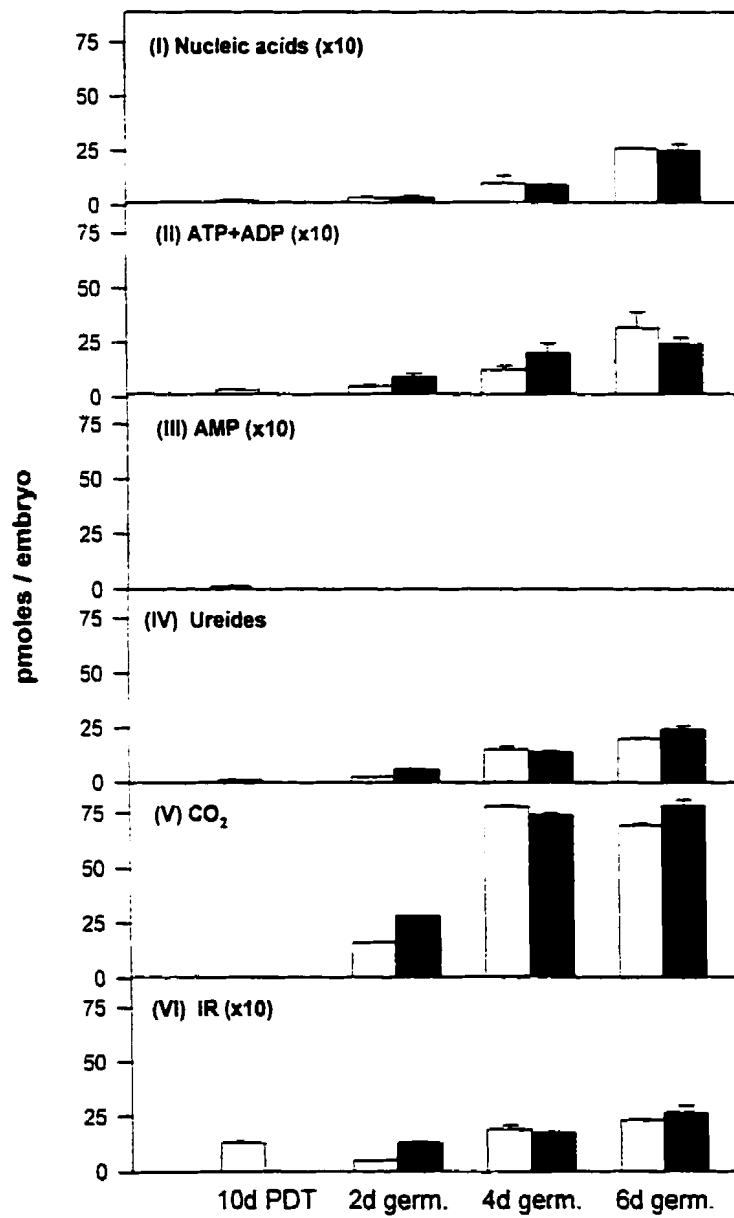
Fig. 5.6. Specific activities of the enzymes involved in the salvage of adenine,

adenosine, and inosine in white spruce somatic embryos germinated in the

absence (open bars) or in the presence (solid bars) of $10^{-4}M$ ascorbic acid (AA).

Activities are expressed as pkat mg^{-1} protein $\pm$ SE. APRT, adenine

phosphoribosyltransferase; AK, adenosine kinase, NPT(adenosine), nucleoside

phosphotransferase measured with adenosine; ARN, adenosine nucleosidase; IK,

inosine kinase; NPT (inosine), nucleoside phosphotransferase measured with

inosine. PDT, partial drying treatment; germ., germination.
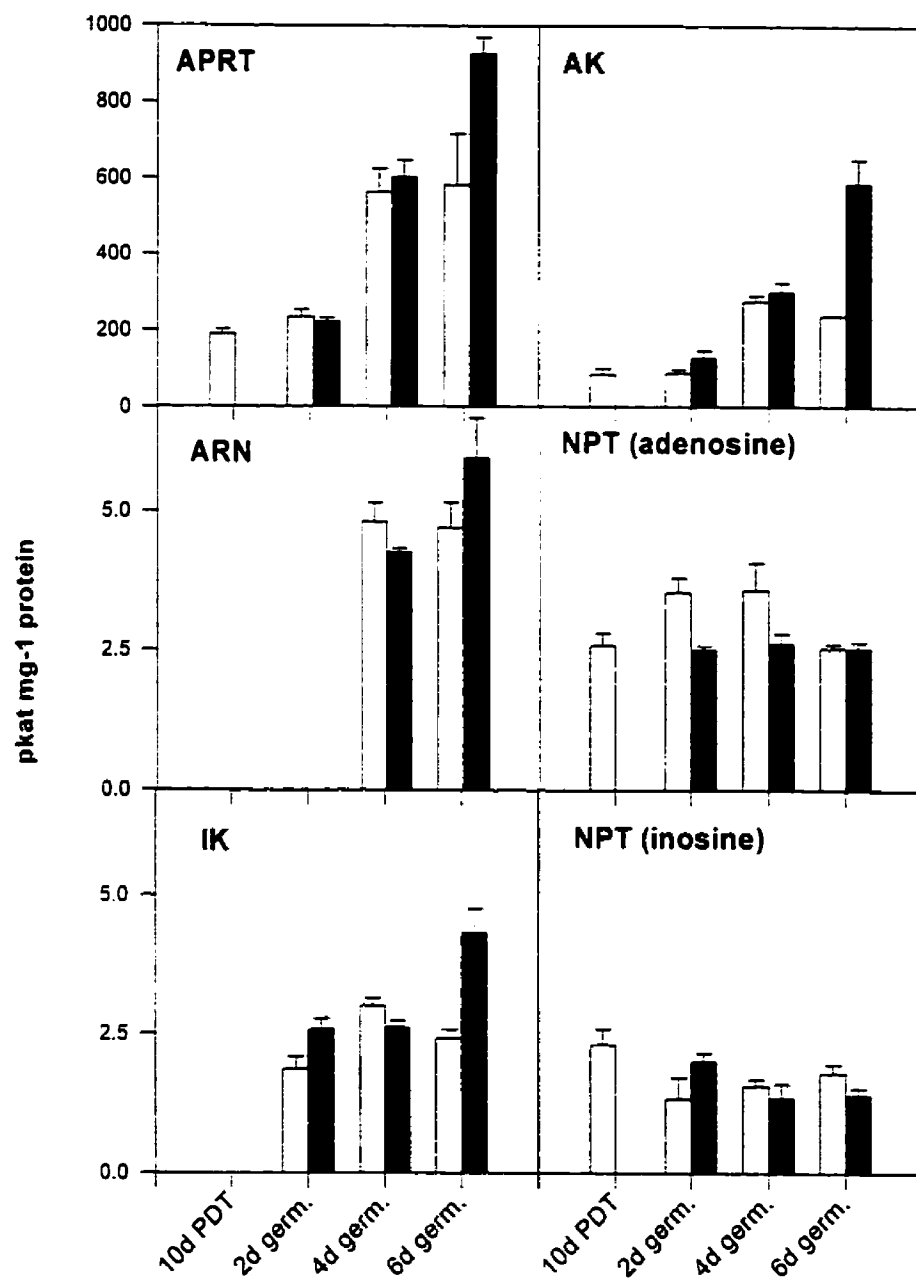
Pyrimidine metabolism

Total uptake of purine intermediates

The total uptake of pyrimidine precursors is shown in Table 5.2. The rates of uptake of uracil, uridine, and orotic acid were lowest in partially dried embryos (10d PDT). and gradually increased during germination. The uptake of uracil and uridine was higher than that observed for orotic acid throughout the period of the experiment. Similar uptake values were observed in both control and AA-treated embryos.

De novo pathway: metabolism of [6-^{14} C]orotic acid

The pyrimidine de novo pathway was investigated by following the metabolic fate of ^{14}C-labelled orotic acid. At the end of the partial drying treatment (PDT) an appreciable proportion of radioactivity from [6-^{14}C]orotic acid was recovered into salvage products. i.e. nucleic acids (0.9 pmol embryo^{-1}) and nucleotides (UTP÷UDP÷UTP) (6.3 pmol embryo^{-1}). A large amount of radioactivity remained in unmetabolized orotate (data not shown), whereas a limited percentage of incorporation was released as CO_2 (Fig. 5.7).

During germination, the percentage of ^{14}C incorporated into the nucleotide and the nucleic acid fractions increased in both control and AA-treated embryos (Fig. 5.7).

Salvage pathway: metabolism of [2-^{14}C]uridine

The salvage pathway of pyrimidine metabolism was investigated by following the metabolic fate of ^{14}C-labelled uridine in germinating embryos. In partially dried embryos (10 d PDT). a large fraction of uridine taken up by the embryos was utilized for nucleotide synthesis. During germination, the amount of radioactivity recovered as salvage products. i.e. nucleic acids and nucleotides, increased equally in both control and AA-treated embryos (Fig. 5. 8). The radioactivity from uridine release as CO_2 was always lower than that recovered as salvage products throughout the first 6 days of germination.

Degradation pathway: metabolism of [2-^{14}C]uracil

At the beginning of the germination period (10 d PDT) a large proportion of [2-^{14}C]uracil, intermediate of the degradation pathway, was released as CO_2 (18.6 pmol embryo^{-1}). Only a limited amount of radioactivity was recovered into salvage products, namely nucleotides and nucleic acids (Fig. 5.9). As germination progressed, the amount of radioactivity from uracil recovered as nucleic acids, nucleotides, and CO_2 increased in both control and AA-treated embryos.

Enzymes of pyrimidine metabolism

Changes in specific activities of the enzymes involved in the pyrimidine salvage and de novo pathways are shown in Table 5.3. Among the enzymes participating in the salvage of uracil and uridine, uridine kinase (UK) gradually declined during the first 6 days of germination, whereas uracil phosphoribosyltransferase (UPRT) remained constant in both control and AA-treated embryos. In partially dried embryos, the specific activity of the former was markedly higher than that of the latter. Very low activity of uridine phosphotransferase was detected throughout the germination period.

The activity of orotate phosphoribosyltransferase, an intermediate enzyme of the pyrimidine de novo pathway, was found to increase after 2 days of germination in both control and AA-treated embryos, whereas the specific activity of the PRPP-producing enzyme, PRPP synthetase, reached its maximum at day 4 and then decreased. No differences in the activity of the enzymes involved in pyrimidine metabolism were observed between control and AA-treated embryos (Table 5.3).

Table 5.2. Uptake of ¹⁴C-labeled pyrimidine precursors by white spruce somatic embryos germinated in the absence (control) or in the presence (AA) of 10⁻⁴M ascorbic acid. Somatic embryos were incubated for 2 hours in the presence of the labeled precursors. Values are expressed as Bq embryo⁻¹±SE. PDT, partial drying treatment; germ., germination.

¹⁴C-precursor	10d PDT	2d germ.		4d germ.		6d germ.	
		Control	AA	Control	AA	Control	AA
[6-¹⁴C]Orotic acid	8.7±1.8	16.6±1.4	13.1±3.3	38.4±2.0	40.5±3.2	28.0±0.6	34.0±5.1
[2-¹⁴C]Uridine	10.6±1.7	64.6±2.5	67.7±2.6	125.3±5.5	135.4±6.0	162.5±5.7	151.5±4.6
[8-¹⁴C]Uracil	18.1±0.7	62.8±11.4	73.3±3.7	202.5±24.9	186.9±1.4	179.5±9.6	190±4.8

Fig. 5.7. Distribution of radioactivity into different labeled fractions of germinating white spruce somatic embryos incubated for 2 hours with [6-^{14}C]orotic acid. Embryos were germinated in the absence (open bars) or in the presence (solid bars) of 10^{-4}M ascorbic acid. Values are expressed as means ± SE. (10x), values are multiplied by a factor of 10. PDT, partial drying treatment; germ., germination.

Orotic acid

(I) Nucleic acids

(II) UTP+UDP+UMP

(III) CO² (10x)

pmoles / embryo

10d PDT   2d germ.   4d germ.   6d germ.
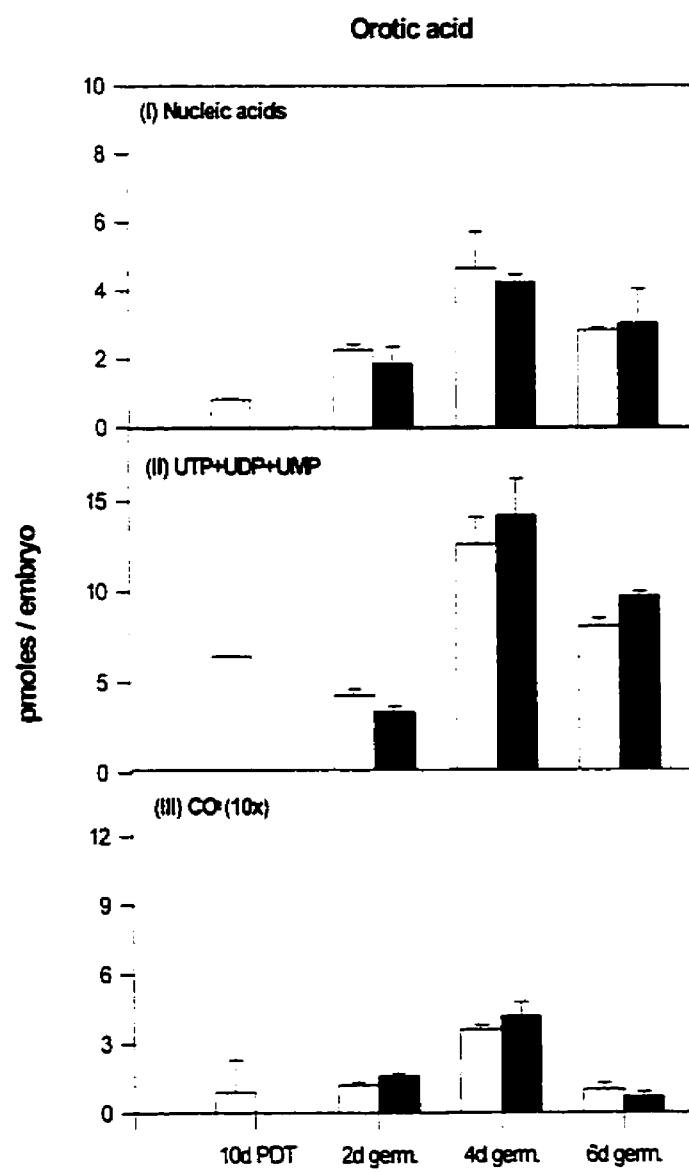
Fig. 5.8. Distribution of radioactivity into different labeled fractions of germinating white spruce somatic embryos incubated for 2 hours with [2-^{14}C]uridine. Embryos were germinated in the absence (open bars) or in the presence (solid bars) of 10^{-4}M ascorbic acid. Values are expressed as means ± SE. PDT, partial drying treatment; germ., germination.

Uridine

(I) Nucleic acids

(II) UTP+UDP+UMP

(III) CO_2

pmoles / embryo

10d PDT   2d germ.   4d germ.   6d germ.
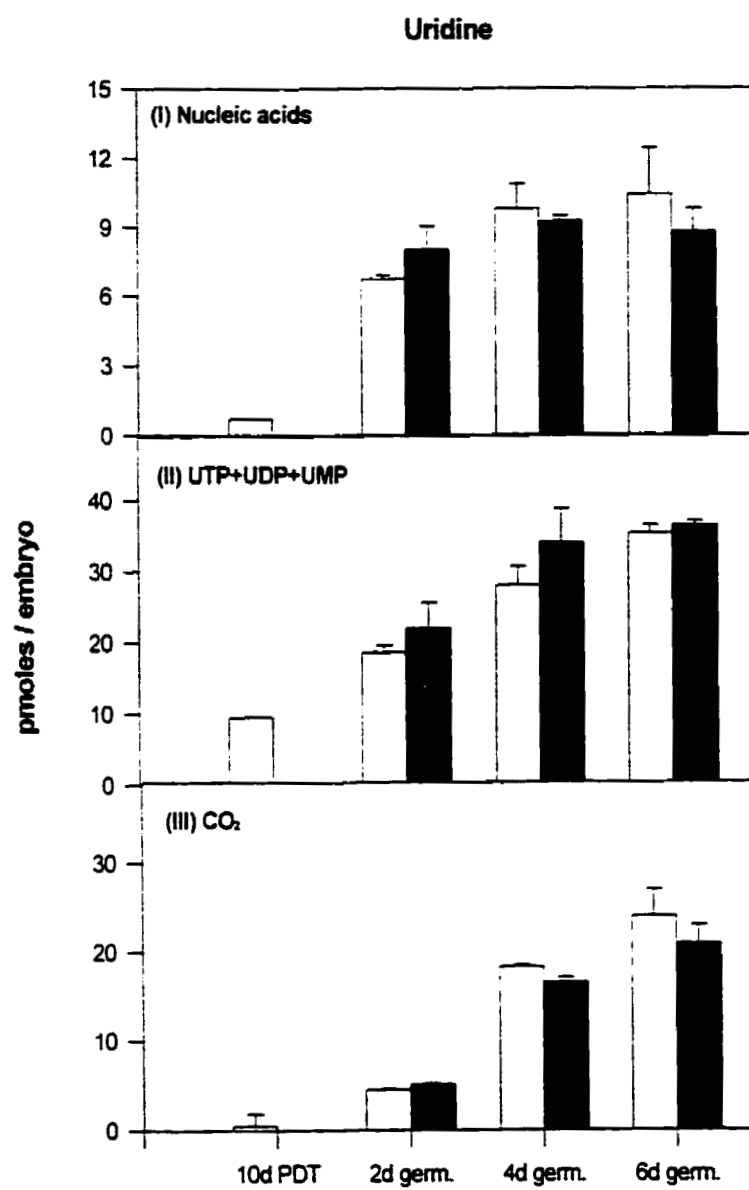
Fig. 5.9. Distribution of radioactivity into different labeled fractions of germinating white spruce somatic embryos incubated for 2 hours with [2-^{14}C]uracil. Embryos were germinated in the absence (open bars) or in the presence (solid bars) of 10^{-4}M ascorbic acid. Values are expressed as means $\pm$ SE. PDT. partial drying treatment; germ., germination.

Uracil

(I) Nucleic acids

(II) UTP+UDP+UMP

pmoles / embryo

(III) CO$_2$

10d PDT   2d germ.   4d germ.   6d germ.
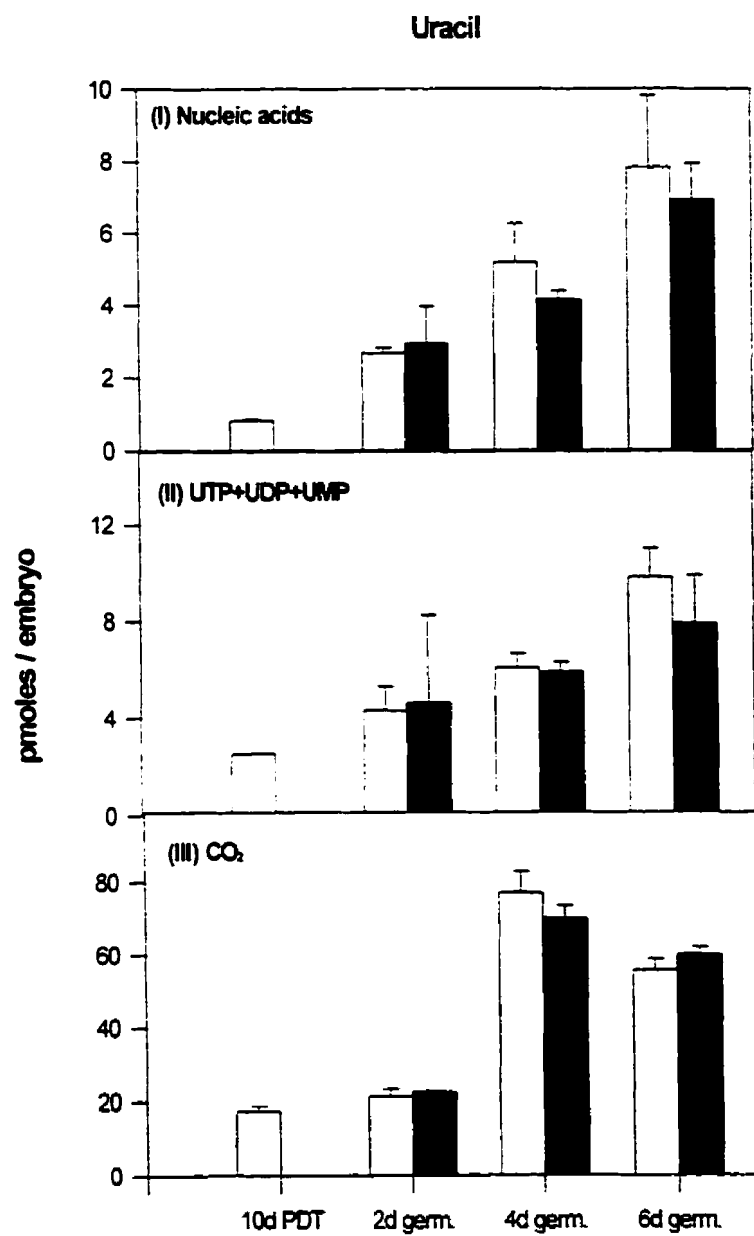
Table 5.3. Specific activities of the enzymes involved in pyrimidine metabolism during germination (germ.) of control (C) and ascorbate (AA)-treated embryos of white spruce. Activities are expressed as pkat mg^{-1} protein ±SE.

Enzyme	10d PDT	2d germ.		4d germ.		6d germ.	
		C	AA	C	AA	C	AA
Uracil phosphoribosyltransferase	1.9±0.3	1.4±0.1	1.7±0.7	2.1±0.2	1.9±0.3	3.0±0.2	2.9±0.3
Uridine kinase	45.7±1.4	12.9±1.2	14.6±2.7	23.8±4.5	18.9±5.0	6.3±0.8	6.0±0.4
Nucleoside phosphotransferase (uridine)	1.4±0.9	1.2±0.1	1.5±0.7	1.2±0.0	0.9±0.6	1.4±0.1	1.4±0.3
Orotate phosphoribosyltransferase	27.6±4.1	21.9±0.9	19.6±2.1	37.9±4.8	38.9±5.2	39.7±3.0	36.5±1.4
PRPP synthetase	27.6±5.5	23.8±3.1	20.7±4.0	40.5±7.5	36.6±6.1	27.1±1.9	24.6±5.0

Discussion

As reported in Chapter 3, exogenous applications of ascorbic acid increased the conversion frequency of somatic embryos of the (E)WS1 line to 58%, compared with 33.5% of the control embryos. To ascertain whether ascorbate applications result in the alteration of nucleotide biosynthesis, ^{14}C-labeled purine and pyrimidine precursors were utilized for tracer experiments in white spruce somatic embryos of the (E)WS1 line germinated in the absence (control) and in the presence (AA-treated) of 10^{-4}M ascorbic acid.

Purine metabolism.

Total uptake

From Table 5.1, it is apparent that the uptake of all the fed purines was extremely low in the partially dried embryos (10 days PDT). This low uptake denotes an overall reduced metabolic activity of white spruce somatic embryos at the end of the drying period. A similar result was also reported in dry *Sinapis arvensis* seeds fed with ^{14}CO$_2$ (Edwards. 1976). After transfer to germination medium. however. a sharp increase in the uptake of [8-^{14}C]adenine. [8-^{14}C]adenosine. and [8-^{14}C]inosine was observed in both control and AA-treated embryos. This increased uptake of purine precursors. also observed in germinating soybean embryonic axes by Anderson (1977). is indicative of a resumption of the overall cellular metabolism following imbibition.

The AA-treated embryos showed higher uptake of both adenine and adenosine compared with the control counterparts throughout the first six days of germination (Table 5.1). This result might be ascribed to changes in plasma membrane properties induced by exogenous applications of ascorbic acid (Asard et al.. 1995; Gonzales-Reyes et al.. 1992; Horemans et al.. 1994). In line with this hypothesis. Gonzales-Reyes et al. (1992. 1994. 1995) reported rapid growth by cell expansion. and accelerated uptake of sugars and nitrates. in onion roots treated with exogenous applications of AA. Similar processes might

regulate the stimulated intake of adenine and adenosine in germinating white spruce somatic embryos in the presence of a large availability of AA.

Salvage pathway of purines

The salvage pathway of purines was investigated by following the metabolic fate of [8-^{14}C]adenine and [8-^{14}C]adenosine (Fig. 5.1). The increasing proportion of [8-^{14}C]adenine and [8-^{14}C]adenosine recovered in nucleic acids and nucleotides (Figs. 5.3 and 5.4) indicates that the ability to synthesize adenylate nucleotides (mainly ADP+ATP) from purine intermediates is low at the end of the partial drying treatment (10 days PDT), but increases at the onset of germination in both control and AA-treated embryos. This increase paralleled the rise in specific activities of the enzymes involved in the purine salvage pathway (Fig. 5.6).

The rate of conversion of adenine and adenosine in nucleotides is modulated by the enzymes adenine phosphoribosyltransferase (APRT) and adenosine kinase (AK). The salvage of adenine is mainly catalyzed by APRT (Fig. 5.1, step 2), which sharply increased after 2 days of germination (Fig. 5.6). Significant levels of this enzyme in germinating *Phaseolus mungo* seeds (Ashihara, 1983), and during dormancy release of peach buds (Lecomte and Le Floc'h, 1999) suggest that APRT may play an active role in ensuring the enlargement of the nucleotide pool during phases of intensive growth.

Biosynthesis of adenylate nucleotides from adenosine can occur either as a single step reaction, via AK (Fig. 5.1, step 1), or by hydrolysis to adenine mediated by adenosine nucleosidase (ARN) (Fig. 5.1, step 3), followed by the addition of a phosphoribosyl group catalyzed by APRT (Fig. 5.1, step 2). The high activity of AK in both control and AA-treated embryos throughout the germination period indicates that the recycling of adenosine occurs primarily through this enzyme. The alternative route of adenosine salvage does not seem to be operative during the first days of germination, because adenosine nucleosidase (ARN) was only detected at day 4 (Fig. 5.6). It is worth noting, however, that the contribution of this enzyme to adenosine salvage was limited compared to that of AK, as shown by its reduced specific activity. ARN, which represents the preferential enzyme of

adenosine salvage in leaves of peach trees (Le Floc'h and Faye. 1995). seems to be strictly associated with the germination processes of white spruce somatic embryos. since its activity was undetected in cultured white spruce cells (Ashihara et al., 2000). during somatic embryo development (Ashihara et al., 2001) and desiccation (Stasolla et al.. 2001c). Similar to our results, ARN was absent in dried *Lupinus luteus* seeds but increased significantly following imbibition (Guranowski and Pawelkiewicz, 1978).

Differences in purine salvage between control and AA-treated embryos were visible 4 days after transfer to germination medium. The larger proportion of radioactivity from [8-^{14}C]adenine and [8-^{14}C]adenosine recovered into the nucleotide (mainly ADP+ATP) fraction of AA-treated embryos (Figs. 5.3 and 5.4). together with the higher activities of APRT and AK (Fig. 5.6). indicates that ascorbate increases the rate of adenine and adenosine salvage during the germination of white spruce somatic embryos. An operative anabolism of adenine and adenosine. also reported in axes and cotyledons of germinating *Phaseolus mungo* embryos (Ashihara. 1983; Nobusawa and Ashihara, 1983), is critical for the enlargement of the nucleotide pool needed to sustain the reactivation of the overall cell metabolism at imbibition. High levels of ATP during the early phases of germination are required not only as building blocks for nucleic acid synthesis, but also as fundamental intermediates participating in many biosynthetic processes. Increasing concentrations of ATP were in fact observed during the early stages of germination in pollen pine (Nygaard. 1973). bacterial spores (Setlow and Kornberg 1970). and soybean embryos (Anderson. 1977). Furthermore. a positive correlation between seed viability and endogenous amount of ATP has been reported in several species (Ching. 1973. 1975). Comparative studies between control and deteriorated soybean seeds. characterized by low percentage of germination. revealed a higher endogenous level of ATP in the former (Anderson, 1977). The author also suggested that the impaired ability to generate ATP, rather then the loss of specific activities of the enzymes involved in biosynthetic processes, is responsible for the reduced germination of the deteriorated seeds. In agreement with this suggestion, the AA-stimulated salvage of adenine and adenosine observed in our study may be necessary to provide the embryos with that surplus of adenylate nucleotides necessary for successful embryo conversion.

Degradation of purines

Purine degradation was investigated by following the metabolic fate of [8-^{14}C]inosine. Most of the radioactivity from [8-^{14}C]inosine was recovered as degradation products. i.e. CO_2 and ureides. in both control and AA-treated embryos. A similar extensive catabolism of inosine was also observed in cultured cells (Ashihara et al.. 2000) and during somatic embryo maturation (Ashihara et al.. 2001) of white spruce. The reduced inosine anabolism is due to the low activity of inosine kinase (IK). responsible for the conversion of inosine to IMP (Fig. 5.1. step 4). and to low levels of nucleoside phosphotransferase (NPT) measured with inosine (Fig. 5.6). Low activities of NPT were also observed in cultured *Catharanthus roseus* cells (Hirose and Ashihara, 1984), where this enzyme was solely responsible for the salvage of inosine, since no IK activity was detected.

Pyrimidine metabolism

From the pyrimidine metabolism studies. it emerges that the de novo. salvage. and degradation pathways are operative in both control and AA-treated embryos. However. contrary to purine metabolism, applications of AA did not alter the metabolic fate of supplied pyrimidine precursors and the activities of the major de novo and salvage enzymes.

Conclusions

Data reported in this study suggest that both purine and pyrimidine metabolism are operative during germination of white spruce somatic embryos. Inclusions of AA in the germination medium were found to affect only purine nucleotide biosynthesis. Comparison of purine metabolism between control and ascorbate (AA)-treated embryos, reveals that both the total uptake and the salvage of adenine and adenosine are significantly

higher in the latter. The extensive anabolism of adenine and adenosine observed in the presence of ascorbic acid is ascribable to a higher activity of the respective salvage enzymes, APRT and AK. Although it was not possible to determine whether the AA-stimulated anabolism of purines is the driving force or the result of the increased embryo conversion, it is clear that AA induces a extensive recycling of purine bases and nucleosides for nucleotide production. Increased amount of nucleotides during the early stages of germination might be necessary to carry out DNA synthesis, associated with the resumption of mitotic activity in the apical meristems of the embryos.

CHAPTER SIX

The effects of ascorbic acid on nucleic acid synthesis of germinating white spruce somatic embryos.

Introduction

Alterations of the endogenous level of ascorbic acid (AA) affect the ability of white spruce somatic embryos to convert into viable plantlets. Exogenous applications of AA increased the conversion frequency of white spruce somatic embryos of the (E)WS1 line (Chapter 3), whereas inhibition of the AA de novo biosynthetic pathway with lycorine, had an opposite effect on the embryos of the (E)WSC line (Chapter 4). These observations, together with the increased salvage of purine precursors in AA-treated embryos (Chapter 5) clearly suggest that cellular AA might be involved in cell division processes. The post-embryonic performance of white spruce somatic embryos is strictly dependent upon the reactivation of the apical meristems at germination. During the early stages of germination, resumption of DNA synthesis, followed by cell division, occurs in both shoot and root apical poles, thus allowing the growth of the embryo. Therefore, a positive correlation between endogenous AA level and resumption of mitotic activity of germinating white spruce somatic embryos seems to exist.

Several studies conducted on both animal and plant systems have documented the participation of AA during DNA replication and cell division processes. Applications of ascorbic acid have been found to amplify tumor promotion in rat bladder carcinogenesis (Fukushima et al., 1988), as well as to promote DNA synthesis in chemically induced forestomach tumors (Shibata et al., 1992). In plants, AA has been found to induce cell division in several systems, including tobacco cultured cells (de Pinto et al., 1999), *Lupinus albus* seedlings (Arrigoni et al., 1997), *Allium cepa* roots (Liso et al., 1984), and corn roots (Kerk and Feldman, 1995). Although the mode of action of AA during the cell cycle is not well understood, AA may be required for the progression of cells through the G_1/S and G_2/M transitions in both onion and pea roots (Liso et al., 1984; Citterio et al.,

1994). Furthermore, quiescent embryo cells speeded up the G_0/G_1 transition during germination in the presence of AA (Citterio et al., 1994). The utilization of AA as a cofactor during the synthesis of hydroxyproline-rich glycoproteins, many of which participate in cell cycle events, further supports these findings (De Gara et al., 1991).

The specific incorporation of labeled thymidine into DNA has been frequently used as a measure of DNA synthesis in both animal (Bianchi et al., 1997) and plant (Kameyama et al., 1985) systems. In plant cells, utilization of thymidine for DNA synthesis is regulated by the activity of the salvage and degradation pathways, which are mutually competitive (Fig. 6.1). An operative salvage pathway is often observed in tissue undergoing intense growth, including germinating wheat grains (Rejman and Buchowicz, 1971) and white spruce zygotic embryos (Stasolla et al., unpublished observation). Therefore, estimation of the activity of thymidine salvage and degradation pathways is indicative of the ability of cells to undergo DNA replication.

The main objective of this study was to determine whether the endogenous levels of AA affect the conversion frequency of white spruce somatic embryos by regulating (1) thymidine metabolism and (2) DNA synthesis in the apical meristems. In order to test this hypothesis, in vivo tracer experiments and autoradiographic studies using radiolabeled thymidine were performed in dissected root and shoot segments of germinating embryos, cultured in the presence of exogenous AA and L.

Materials and methods

Plant material

Establishment and maintenance of white spruce cell cultures

Establishment and maintenance of the (E)WSC line, utilized in this study, were those described in Chapter 4.

Fig. 6.1. Proposed salvage and degradation pathways for thymidine in root and shoot segments of white spruce somatic embryos. Enzymes corresponding to the numbered reactions are: (1) deoxynucleoside-monophosphate kinase and/or TMP kinase; (2) nucleoside diphosphate kinase; (3) DNA polymerase; (4) thymidine phosphorylase and/or nucleosidase; (5) dehydroxyuracil dehydrogenase; (6) dehydropyrimidinase; (7) β-ureidopropionase; (8) unknown enzyme. NPT. nucleoside phosphotransferase; TMP, thymidine monophosphate; TDP. thymidine diphosphate; TTP. thymidine triphosphate; TK. thymidine kinase.
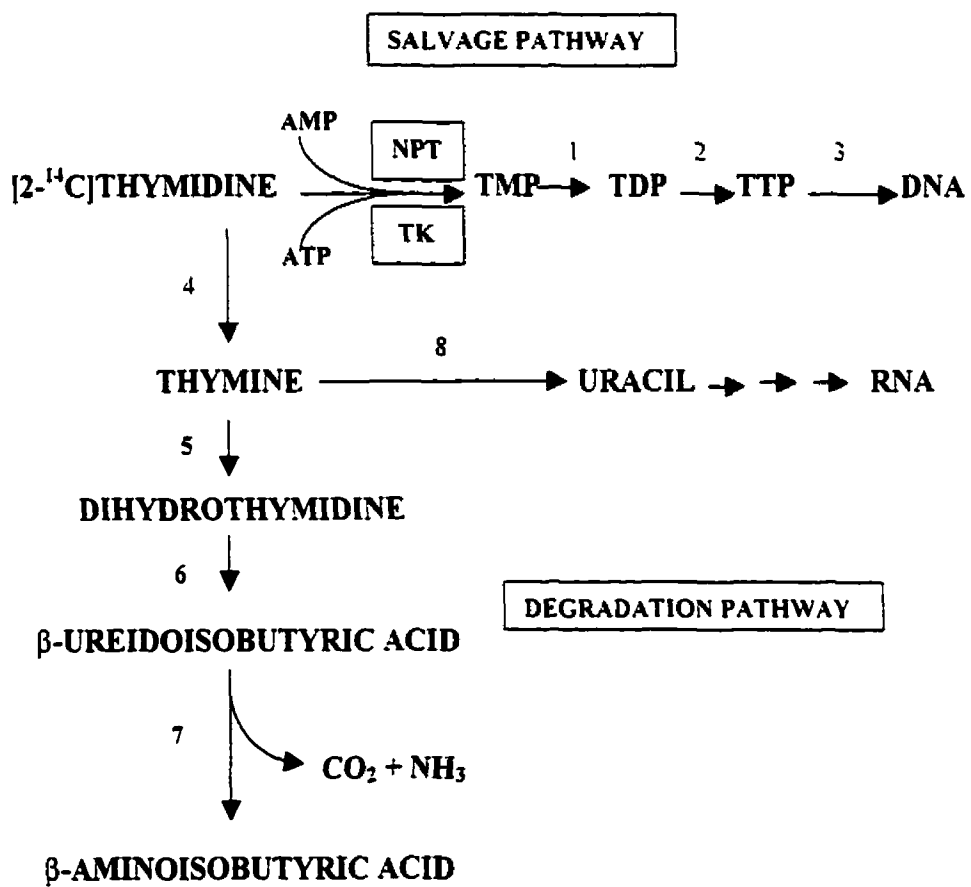
Somatic embryo development, partial drying treatment, and germination

Culture conditions for somatic embryo maturation and germination were similar to those described in Chapter 4.

Alterations of the endogenous AA levels and tissue sampling

Applications and concentrations of exogenous ascorbic acid (AA) and lycorine (L), utilized for altering the endogenous AA level of germinating embryos, have been described in Chapter 4. For this experiment, however, root and shoot apices (1 mm in length) were dissected from the embryos at day 6 and day 12 after germination and utilized for tracer experiments and autoradiographic studies.

Chemicals

[2-^{14}C]thymidine (specific activity, 1.85 MBq. μmol^{-1}), and [methyl-^{3}H]thymidine (specific activity, 2.2MBq. μmol^{-1}) were obtained from Moravek Biochemicals Inc. (Brea, CA, USA).

Metabolism of ^{14}C-thymidine

Administration of labeled thymidine was carried out as previously described (Chapter 5). Tissue (60 root or shoot segments) was incubated with 10 μM [2-^{14}C]thymidine for 2 hours at 22° C. Extraction and analysis of labeled metabolites were performed as described in Chapter 5.

Enzyme assays

The extraction procedure for the enzyme assay was similar to that described in

Chapter 5. For each enzyme assay 35 µL of desalted extract was incubated for 2, 5, and

10 min at 30° C in the following reaction mixtures (total volume 100 µL).

I) Thymidine kinase (TK). This was determined in a reaction mixture containing

30 mM HEPES-NaOH buffer (pH. 7.6), 10 mM $MgCl_2$, 1 mM DTT, 3.75 mM ATP, and

45 µM of [2-^{14}C]thymidine) (Kameyama et al., 1985).

II) Nucleoside phosphotransferase (NPT). The reaction mixture was similar to

that utilized for thymidine kinases, except that ATP was replaced by 3.75 mM AMP

(Kameyama et al., 1985).

Termination of reaction and visualization of the product on TLC plates was

carried out as described in Chapter 5. More than three independent experiments were

performed. Specific activity of the enzymes was expressed as pkat mg^{-1} protein.

Statistical analysis

Statistical analysis was carried out as described in Chapter 2.

Autoradiography

Autoradiography of [methyl-^{3}H]thymidine was carried out as described by Yeung

(1984b). Dissected root and shoot segments were incubated in the presence of [methyl-

^{3}H]thymidine (1.5 µCi/ml) for 24 hours, and washed in distilled water. After fixation in

FAA (formaldehyde-ethanol-acetic acid [10%-50%-5%]), the samples were dehydrated in

an ethanol series, and embedded in Historesin. Serial longitudinal sections were cut at a

thickness of 4 µm. The selected slides were dipped into photographic emulsion (Kodak

NTB2) diluted to half the original concentration using warm distilled water. After a 10-

day exposure at 4° C in the dark, the slides were developed and fixed using Kodak D-19

developer and in Kodak fixer respectively, according to Yeung (1984b). The sections were stained in a 0.1% TBO (toluidine blue O solution) buffered in benzoate (pH 4.4) for 3 min. The preparations were examined and photographed with a Leitz Aristoplan light microscope.

Results

The effect of AA on DNA synthesis was studied by following the metabolic fate of exogenously supplied ^{14}C-thymidine in root and shoot segments of white spruce somatic embryos germinated in the presence of ascorbic acid (AA) and/or lycorine (L). As reported in Chapter 4, treatments with L reduced the conversion frequency of the embryos to 21.1%, compared to 88.5% of control (C) embryos (Table 4.1, Chapter 4), as well as the endogenous AA content (Fig. 4.1, Chapter 4). Such effects were partially reversed when AA was added to lycorine (AA+L). Applications of AA did not enhance the conversion frequency of the embryos, but increased the endogenous level of ascorbic acid (Table 4.1 and Fig. 4.1, Chapter 4).

Total uptake of ^{14}C-labeled thymidine

At day 6, the total uptake of exogenously supplied thymidine was similar for all treatments (between 16 and 19 pmol per shoot segment and between 43 and 53 pmol per root segment (Fig. 6.2). At day 12 the amount of thymidine taken up by the shoot segments did not change significantly among the treatments. In control roots, as well as in AA- and AA+L-treated roots, the total uptake declined below 35 pmol explant^{-1}, whereas it remained high for L-treated roots. As revealed by statistical analysis, significant differences in uptake values were observed between control and L-treated roots at day 12 (Fig. 6.2).

Fig. 6.2. Total uptake of labeled thymidine in shoot (**A**) and root (**B**) segments of white spruce somatic embryos. Values are mean $\pm$ SE of three independent experiments. C. control: L. lycorine: AA. ascorbic acid: AA+L. ascorbic acid plus lycorine.
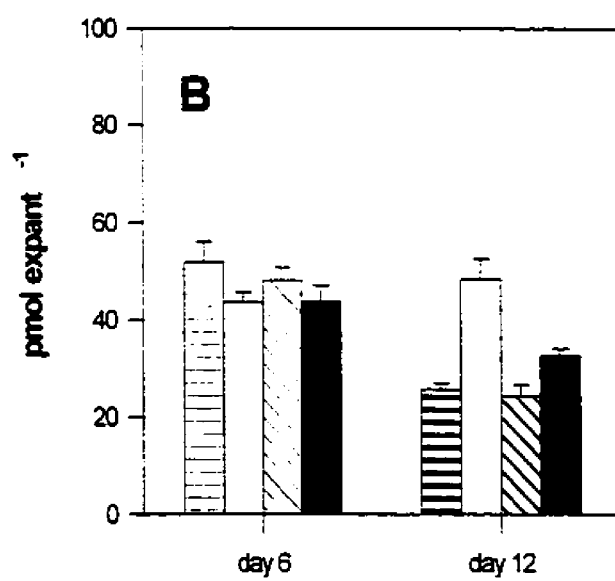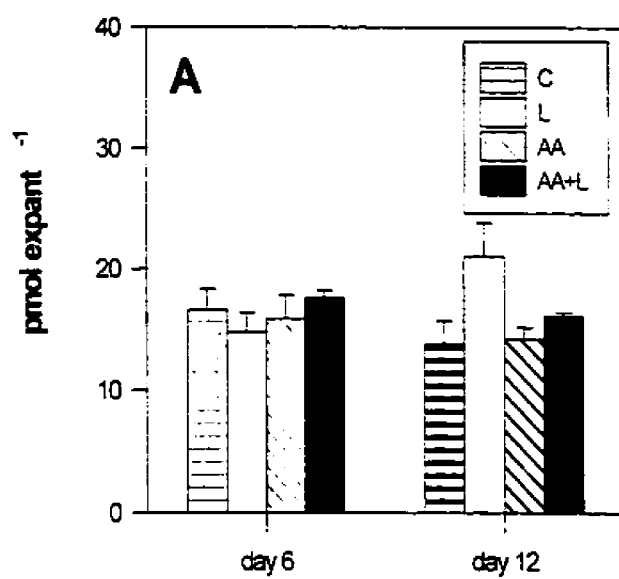
Metabolic fate of ^{14}C-labeled thymidine

A large percentage of fed thymidine taken up by both root and shoot segments was catabolized to degradation products. i.e. CO_2 and β-ureidoisobutyric acid (Fig. 6.1 and Table 6.1). At day 6, more than 70 % and 0.7% of radioactivity from ^{14}C-labeled thymidine taken up by the root segments was recovered as CO_2 and β-ureidoisobutyric acid respectively. At day 12, however, the percentage of radioactivity released as CO_2 decreased in C, and in AA-, and AA+L- treated root segments, where a larger portion of thymidine remained unmetabolized (data not shown), whereas it remained high in L-treated root segments. The difference in $^{14}CO_2$ released from control and L-treated root segments at day 12 was statistically significant. Extensive degradation of fed thymidine was also observed in shoot segments. At both day 6 and day 12. more than 66% of radioactivity from [2-^{14}C]thymidine was released as CO_2 in all treatments (Table 6.1).

Only a small fraction of thymidine was salvaged to nucleic acids (DNA+RNA) and nucleotides (TMP. TDP, and TTP) (Fig. 6.1. and Table 6.2). Compared to the control counterparts. the amount of radioactivity from [2-^{14}C]thymidine recovered as salvage products was significantly lower in L-treated root segments at both day 6 and day 12. Intermediate values of thymidine salvage were observed in AA+L-treated roots (Table 6.2). Compared to the control, the incorporation of thymidine into the nucleic acid fraction of AA-treated roots was significantly higher at both day 6 and day 12.

In shoot segments. lycorine (L) treatments resulted in the lowest utilization of thymidine for nucleic acid synthesis at day 12. No major differences in thymidine salvage were observed among the other treatments (Table 6.2).

Table 6.1. Incorporation of ^{14}C-labeled thymidine into the two major degradation fractions of root and shoot segments of white spruce somatic embryos. Values $\pm$ SE are expressed as percentages of total incorporation. C. control; L, lycorine; AA. ascorbic acid; AA+L. ascorbic acid plus lycorine.

	ROOT		SHOOT	
	day 6	**day 12**	**day 6**	**day 12**
CO_2				
C	70.7±8.0	52.9±3.9	75.4±4.5	76.1±1.5
L	78.1±6.7	81.4±3.6	71.6±10.4	73.0±6.5
AA	73.3±9.6	48.1±1.8	68.4±0.9	66.3±3.2
AA+L	77.4±1.3	62.9±2.6	75.5±7.4	73.1±3.6
β-ureidoisobutyric acid				
C	1.5±0.1	1.4±0.4	1.3±0.2	0.8±0.2
L	1.1±0.1	0.7±0.7	0.9±0.2	0.4±0.2
AA	1.2±0.1	1.4±0.2	1.4±0.1	0.6±0.0
AA+L	0.7±0.3	1.1±0.2	1.1±0.1	0.2±0.1

Table 6.2. Incorporation of ^{14}C-labeled thymidine into the different salvage products of root and shoot segments of white spruce somatic embryos. Values ± SE are expressed as percentages of total incorporation. C. control; L. lycorine; AA. ascorbic acid; AA+L. ascorbic acid plus lycorine.

	ROOT		SHOOT	
	day 6	day 12	day 6	day 12
NUCLEIC ACIDS				
C	1.7±0.2	4.6±0.3	1.6±0.2	4.9±0.3
L	0.8±0.1	0.4±0.1	1.9±0.1	1.5±0.2
AA	3.5±0.2	7.4±0.3	2.1±0.1	5.7±0.3
AA+L	1.6±0.1	3.6±0.2	2.1±0.3	4.2±0.1
NUCLEOTIDES				
C	2.0±0.2	1.6±0.2	3.7±0.1	3.5±0.2
L	1.7±0.1	0.7±0.2	3.7±0.3	3.9±0.4
AA	2.3±0.2	1.2±0.1	3.8±0.7	4.5±0.3
AA+L	1.8±0.1	2.1±0.3	3.4±0.3	3.8±0.1

Enzyme assays

The activities of the two thymidine salvage enzymes, thymidine kinase (TK) and nucleoside phosphotransferase (NPT), measured with thymidine as a substrate and AMP as a phosphate donor, were detected in both shoot and root segments (Figs. 6.1 and 6.3). At day 12, the activity of TK was lowest in L-treated shoot and root segments, whereas similar activities of this enzyme were measured for the other treatments. The activity of NPT was always lower compared to that of TK (Fig. 6.3).

Autoradiographic studies

At day 6, both control and AA-treated embryos where characterized by the presence of an emerging shoot primordium and an elongated radicle. Many of the cells within the shoot and root apices were heavily labeled with [methyl-^{3}H]thymidine (Fig. 6.4 A-D). Shoot primordium and radicle emergence where delayed in both L- and AA+L-treated embryos. In these embryos only a few meristematic cells were able to incorporate thymidine in their nuclei (Fig. 6.4 E-H).

After 12 days of germination, root and shoot continued to develop in both control (C) and AA-treated embryos. Although less pronounced than that observed at day 6, incorporation of thymidine in the cells of the shoot apical region occurred in both treatments. Accumulation of label was also observed in root cells, especially those of AA-treated embryos (Fig. 6.5 A-D). No incorporation of thymidine was found in shoot and root cells of L-treated embryos, which were unable to develop further (Fig. 6.5 E, F). In contrast, AA+L treated embryos were able to incorporate thymidine (Fig. 6.5 G, H). Throughout the course of the experiment both shoots and roots of AA+L-treated embryos were more labeled than those of the L-treated counterparts.

Fig. 6.3. Specific activity of the two major salvage enzymes: thymidine kinase (TK) and nucleoside phosphotransferase (NTP), in root and shoot segments of white spruce somatic embryos. Values. expressed as pkat mg^{-1} protein. are means $\pm$ SE of three independent experiments.

ROOT

SHOOT

TK

TK

NTP

NTP

Units (pkat mg⁻¹ protein)

Fig. 6.4. Autoradiographs showing the incorporation of ^{3}H-thymidine in shoot and root apices of white spruce somatic embryos 6 days after germination. The embryos were incubated for 24 hours in the presence of [methyl-^{3}H-thymidine (1.5 μCi/mL). Incorporation of labeled thymidine into the nuclei of shoot and root apices could be found in all treatments at this time. Shoot and root of control (**A**, **B**) and AA-treated (**C**, **D**) embryos were mitotically active. as indicated by a large number of labeled nuclei. On the contrary. the shoots and roots of L- (**E**, **F**) and AA+L- (**G**, **H**) treated embryos had fewer nuclei labeled. All scale bars = 40 μm.
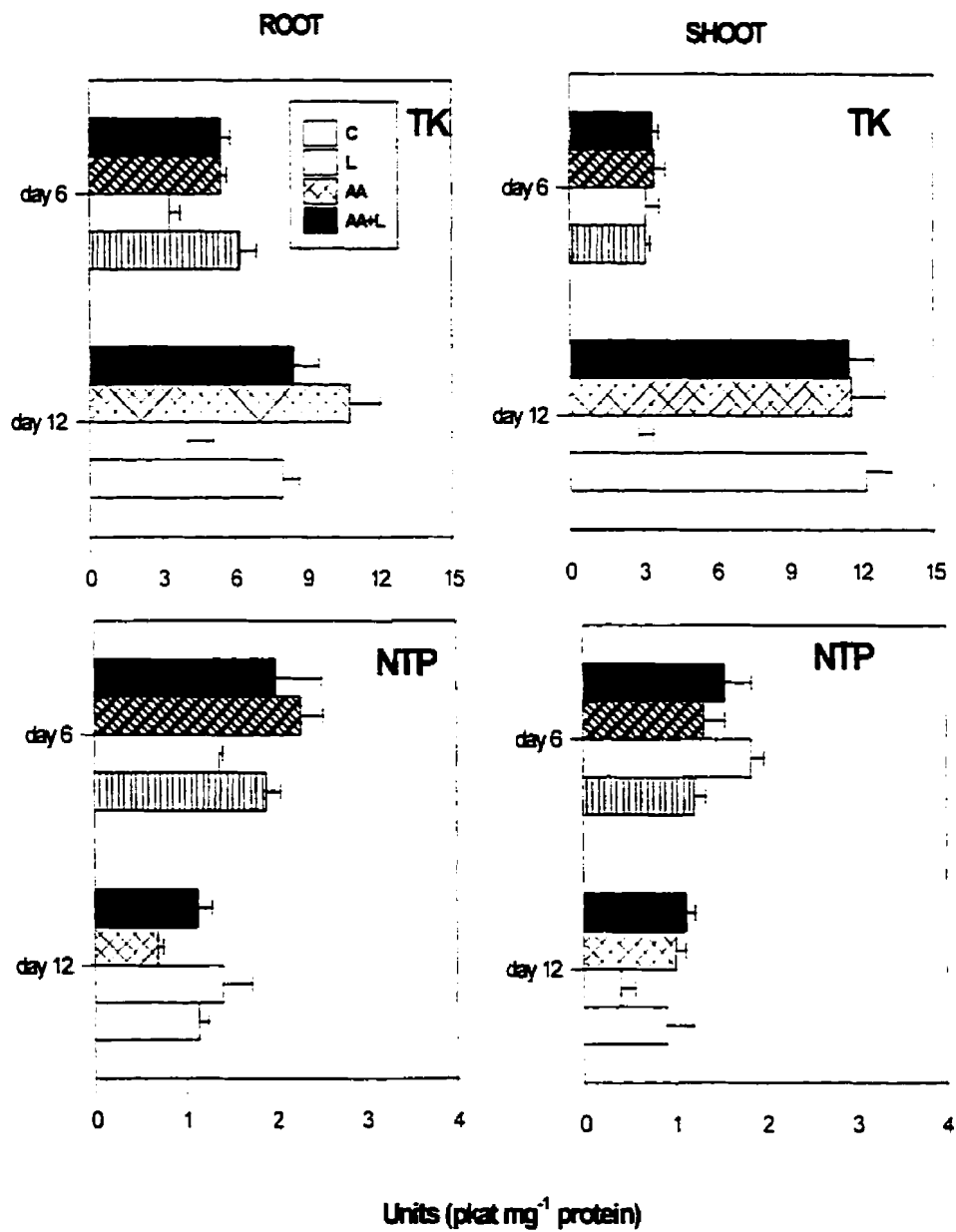
Fig. 6.5. Autoradiographs showing the incorporation of ^{3}H-thymidine in shoot and root apices of white spruce somatic embryos 12 days after germination. The embryos were incubated for 24 hours in the presence of [methyl-^{3}H-thymidine (1.5 µCi/ml). Both shoots and roots of control (**A, B**) and AA-treated (**C, D**) embryos incorporated labelled thymidine in the nuclei. Little incorporation was observed in both shoot and root segments of L-treated embryos (**E, F**), whereas mitotic activity was present in both shoot and root cells of AA+L-treated embryos (**G, H**). All scale bars = 40 µm.
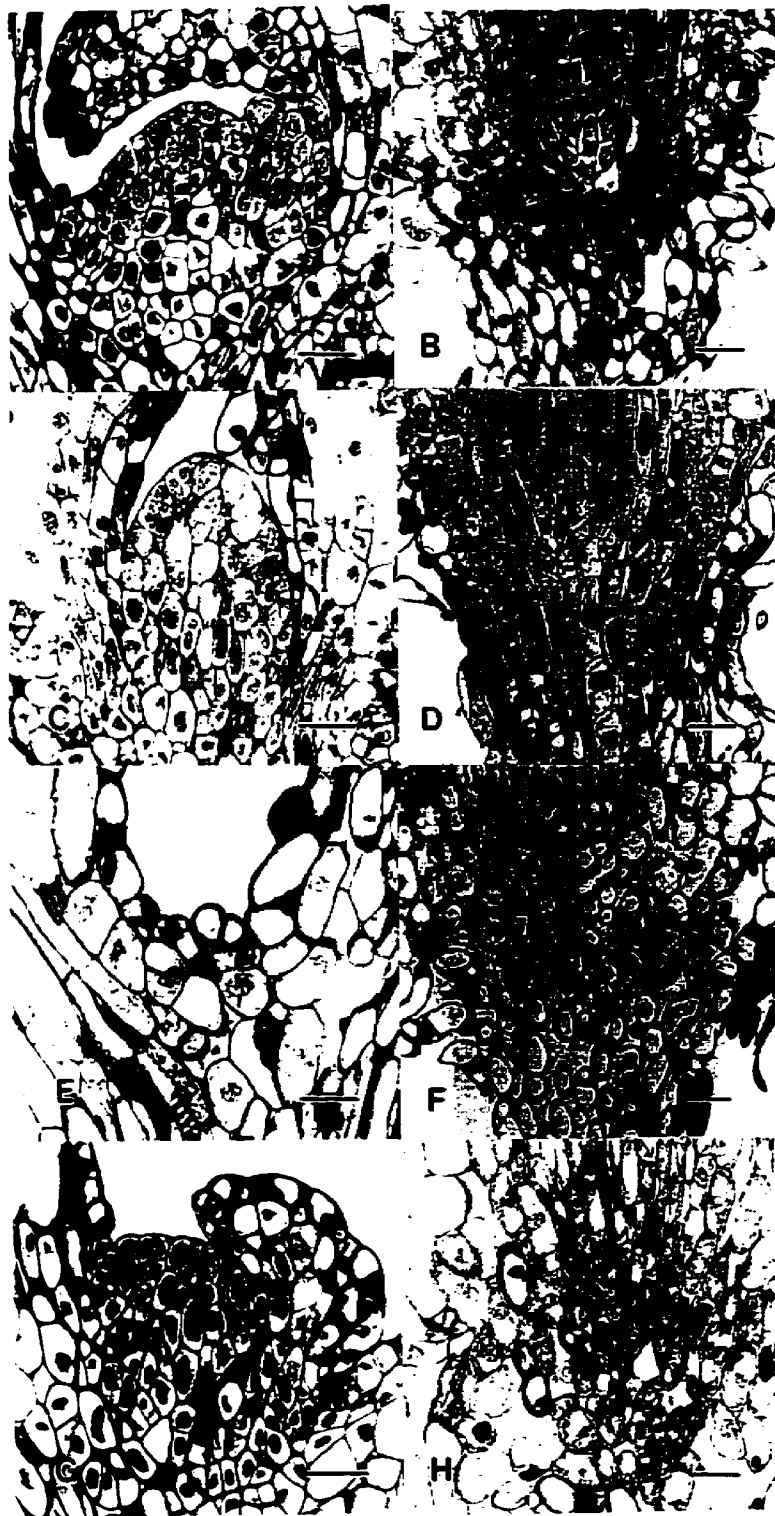
Discussion

In order to determine how changes in the endogenous level of AA affect nucleic acid synthesis, thymidine metabolism was investigated in root and shoot segments of white spruce somatic embryos germinated in the presence of AA, and/or L. As shown in Fig. 6.2, similar uptake values were observed in both root and shoot segments for all treatments, except after L applications at day 12. At day 12, in fact, thymidine uptake in L-treated shoot and root segments was the highest. This result is possibly due to the fact that, besides reducing growth. exogenous applications of lycorine caused a swelling of the apical regions of the embryos, thus increasing the amount of thymidine taken up by each segment. If expressed as pmol gr^{-1} fresh weight. in fact. no differences in total uptake were observed among treatments (data not shown).

As documented by the tracer experiments. a large fraction of thymidine taken up by the dissected roots and shoots was degraded. as little was salvaged for nucleotide and nucleic acid synthesis (Table 6.1 and 6.2). Large catabolism of fed thymidine was also reported in sugarcane cells in suspension cultures (Lesley et al., 1980), as well during maturation of white spruce somatic embryos (Stasolla et al.. unpublished observation). Although first reported in 1961 (Evans and Axelrod. 1961), the mechanisms of thymidine degradation during radioactive labeling experiments in plants have not been fully investigated. From these results. it appears that the catabolic pathway of thymidine proceeds through thymine. dihydrothymine. β-ureidobutyrate and then. with loss of the ^{14}C label as CO_2, as unlabeled β-aminoisobutyric acid (Table 6.1). A similar thymidine catabolic pathway was also documented in carrot suspension cultures (Slabas et al.. 1980) and in rape seedlings (Evans and Axelrod. 1961).

Data emerging from this study suggest that in roots and shoots of germinating white spruce embryos, the activities of the salvage and degradation pathways. as well as DNA synthesis, appear to be related to the cellular AA level. When the endogenous AA level of the embryos is experimentally lowered by lycorine, the activities of the degradation and salvage pathways measured in root segments increase and decrease.

respectively. These effects are reversed if the cellular AA content is maintained at high levels, as observed in AA+L-treated embryos. Similarly, the low thymidine salvage. measured in shoot segments of L-treated embryos, can be increased. close to control levels, by AA+L treatments (Tables 6.1 and 6.2). The poor utilization of thymidine for nucleic acid synthesis, observed in L-treated root and shoot segments. is also supported by autoradiographic studies. Mitotic activity, as estimated by the accumulation of silver grains. is low in L-treated embryos, whereas it is high in both roots and shoots of AA+L-treated embryos (Figs. 6.4 and 6.5). Regulation of the cell cycle by cellular AA is well documented in literature. Liso et al. (1984) reported that lycorine induced a profound inhibition of cell division in the G_1 phase in onion root meristem. They also reported that treatments of onion roots with AA caused quiescent cells to undergo DNA synthesis. and they suggested that a higher ascorbate level reduced the duration of the cell cycle by shortening the G_1 phase (Liso et al.. 1988). This interpretation was also substantiated by additional studies. which documented a positive correlation between endogenous AA level and cell proliferation (Citterio et al.. 1994: Kerk and Feldman. 1995: Arrigoni et al.. 1997). Despite these findings, however. to date no convincing evidence is available on the molecular mechanisms behind this effect. From the present study it appears that high levels of cellular AA may be required for maintaining a high activity of TK. The activity of this enzyme, in fact, is decreased in L-treated shoot and root segments (Fig. 6.3). Thymidine kinase activity is required for the conversion of thymidine to TMP, the first step towards the synthesis of DNA (Fig. 6.1). The participation of TK during DNA replication has been well documented. Although the activity of this enzyme was not detected in several systems. including *Phaseolus mungo* seedlings (Kameyama et al. 1985) and potato extracts (Arima et al.. 1971). possibly due to the dilution effect caused by the heterogeneous population of cells found in these tissues. TK activity appears to be strictly related to DNA synthesis. Hotta and Stern (1963) demonstrated a precise switching on of the TK activity in developing lily microspores just prior to an increase in DNA content of the cells. Similarly, in bacterial and other animal systems, TK activity has been shown to be closely associated with the biochemical events culminating in DNA

synthesis and cell proliferation. In synchronized CEM cells, in fact, the activity of this enzyme was low outside the S phase, and increased just after the entry of the cells in S phase (Bianchi et al., 1997). It is therefore suggested that the activity of TK, regulated by the availability of AA in the system, may determine the fate of thymidine. A low enzymatic activity, as measured in L-treated embryos, would result in extensive thymidine catabolism and poor salvage and DNA synthesis. An opposite utilization of thymidine is observed in AA+L-treated embryos, which have higher levels of endogenous AA and higher TK activity. It must be mentioned, however, that exogenous applications of ascorbic acid alone (AA) do not increase the activity of TK (Fig. 6.3), as well as the percentage of thymidine salvaged in shoot segments (Table 6.2). This can be ascribed to the already high endogenous level of AA present in control embryos. As mentioned in Chapter 4, in fact, the cellular AA is higher in the embryos of the (E)WSC line, utilized in this experiment, than in the embryos of the (E)WS1 line, for which applications of AA improve their post-embryonic performance (see Chapter 3).

The involvement of AA in the modulation of enzymes required for DNA synthesis has also been tentatively suggested in previous investigations. Although without any direct evidence, Citterio et al. (1994) proposed that ascorbate may increase the activity of deoxyribonucleotide reductase, an Fe^{2+}-requiring enzyme responsible for the conversion of nucleotides to deoxyribonucleotides, possibly through the release of iron from the storage protein phytoferritin. Deoxyribonucleotide reductase, as also observed for TK, is an S-phase specific enzyme (Bianchi et al., 1997). This finding further substantiates the hypothesis that AA may modulate the activity of major key enzymes of thymidine metabolism, thus regulating DNA synthesis and cell division.

In conclusion, by experimental manipulations of the endogenous AA content it has been demonstrated that AA is an important metabolite involved in the cell cycle progression of meristematic cells of white spruce somatic embryos. When the endogenous AA content is lowered by lycorine, thymidine degradation is increased in root segments, whereas thymidine salvage and DNA synthesis is reduced in both shoots and roots. These effects can be reversed by applications of exogenous AA. Therefore, it is suggested that

cellular AA may affect DNA replication by regulating the rate of thymidine catabolism and anabolism, possibly through the modulation of the activity of TK.

CHAPTER SEVEN

Summary and future studies

One of the most fundamental processes to occur during the early stages of germination is the reactivation of the shoot and root apical meristems. In white spruce, such a process, normally occurring in germinating zygotic embryos is not always observed in their somatic counterparts. Specifically, the resumption of mitotic activity in meristematic cells of somatic embryos is strictly genotype dependent, as different percentages of conversion are often reported in different cell lines. Thus, treatments that improve embryo conversion could have important practical implications.

In the past few years a lot of attention has been directed towards the role played by ascorbic acid (AA) in fundamental cellular processes, including cell division. This metabolite, commonly present at relatively high concentration in both symplastic and apoplastic compartments has been found to stimulate cell proliferation in both plant and animal systems (see Chapter 1). Thus, it was hypothesised that AA plays an important role during the embryogenic process of white spruce and that alterations in the endogenous AA level affect the ability of the somatic embryos to convert into viable plantlets at germination.

Studies on AA metabolism conducted in Chapter 2 have revealed that AA is needed to carry out important morphogenic events characterized by active cell proliferation, including embryo development and germination. Furthermore, differences in AA metabolism were also observed between zygotic and somatic embryos. During the late stages of embryo maturation, characterized by the naturally occurring desiccation period in zygotic embryos and by the imposed partial drying treatment in the somatic counterparts, the ability to synthesize AA de novo decreased in the former and increased in the latter. In addition, the activity of one of the major AA-recycling enzymes, dehydroascorbate reductase (DHAR) was only detected in zygotic embryos. Therefore, the in vitro environment may alter the overall AA metabolism of the embryos, affecting

their post-embryonic performance. Additional comparative studies between somatic embryos of different cell lines also indicated that during germination, a positive correlation between cellular AA content and conversion frequency exists. Specifically, the embryos of the (E)WS1 line, characterized by a low percentage of conversion, had lower levels of endogenous AA, compared to those measured in the embryos of the (E)WS2 line, which had a higher conversion frequency. Based on these observations, it was proposed that experimental manipulations of the germination medium that increase the cellular AA, would enhance the post-embryonic performance of the embryos.

As documented in Chapter 3, inclusions of AA during germination doubled the number of embryos that were able to convert into viable plantlets. Ascorbic acid (AA) had a more pronounced effect on shoot conversion than on root emergence. Structural studies also revealed that applications of AA were able to induce mitotic activity in poorly organized shoot apical meristems, thus promoting the formation of one or more shoot primordia from the original apical meristem or from the base of the cotyledons. The promotive effect of AA on shoot formation, also described during tobacco organogenesis (Joy et al., 1988), is likely due to the reduced accumulation and/or cross-linking of phenolic compounds within the meristematic cells of AA-treated embryos. As shown in Chapter 3, increased deposition of phenolics in the shoot apical meristems of control embryos always preceded meristem abortion.

The idea that cellular AA might regulate the cross-linking of phenolic substances by modulating the activity of cellular peroxidases was tested in Chapter 4. Through experimental manipulation of the cellular AA, it has been demonstrated that a lowering of the endogenous AA level results in increased activities of both guaiacol and ferulic acid peroxidases, as well as an increased accumulation of phenolics in the meristematic cells at the shoot pole. Opposite results were obtained when the endogenous AA content of the embryos was increased. It was therefore concluded that AA may be involved in the reduction of cell wall cross-linking, by modulating the activities of several peroxidases. The inhibitory effect of AA on the in vivo and in vitro activities of both guaiacol and ferulic acid peroxidases, both of them involved in the cross-linking of the cell wall

components, substantiates this idea. The AA-prevention of cell-wall stiffening would be necessary to allow cell elongation and division of the meristematic cells.

The effect of AA on cell division was tested by investigating nucleotide and nucleic acid biosynthesis during germination. As documented in Chapter 5. applications of AA resulted in an increased ability of the embryos to utilize (salvage) purine bases and nucleosides for nucleotide and nucleic acid synthesis. The activities of the salvage enzymes of adenine and adenosine, adenine phosphoribosyltransferase (APRT) and adenosine kinase (AK), were in fact higher after AA applications. The promotive effect of AA on purine salvage is critical for germination, as it provides the embryos with a large availability of nucleotides, before the reactivation of the de novo biosynthetic pathway. Inefficient production of purine nucleotides from adenine and adenosine may. in fact. be the cause of unsuccessful conversion of control embryos. Besides being utilized for bio-energetic processes. increasing production of purine nucleotides in AA-treated embryos may be necessary to sustain DNA synthesis and cell division during germination.

As reported in Chapter 6. cellular AA seems to play a direct role in the regulation of DNA replication and cell division of meristematic cells of both root and shoot apices. Studies on thymidine metabolism and autoradiographic techniques have revealed that high levels of AA increase the salvage of thymidine by modulating the activity of the respective salvage enzyme thymidine kinase (TK) in both shoot and root poles. Opposite results were observed when the endogenous AA was experimentally lowered in the germinating embryos.

In conclusion, the findings of this thesis clearly indicate that AA plays different roles during the germination of white spruce somatic embryos. The complexity of AA metabolism and the participation of AA in basic cellular processes, including cell expansion and division, make any attempt to identify its mode of action elusive. As such, it is not surprising that despite the interest directed towards this metabolite in both animal and plant systems, to date there is not much information concerning the molecular mechanisms of its action. This thesis, however, represents one of the most

comprehensive studies on the possible involvement of AA on cell division processes. Furthermore, these results also provide valuable insights into the physiological aspects of meristem reactivation at germination. This information could be utilized for improving the conversion process of somatic embryos.

The findings emerging from this work provide the framework for many future investigations.

1) It would be interesting to determine how AA affects the expression pattern of other proteins involved in the cell cycle, including proliferating cell nuclear antigen (PCNA), a component of the RNA polymerase machinery, or cyclins.

2) As cellular AA seems to be involved with cell division, it would be useful to determine whether changes in endogenous AA affect gene expression by modulating DNA methylation. HPLC methods for measurement of methylated cytidine and anti-methylation drugs are currently available.

3) The involvement of the reduced (AA) and oxidized (DHA) forms in the control of the cell cycle would further clarify the physiological significance of the AA/DHA ratio observed during morphogenic events. Does a high AA/DHA ratio represent a specific sensor for cell division and/or for other cellular events? For this purpose, a synchronized cell culture system, such as tobacco BY-2 cells will be useful.

3) As the AA-DHA redox pair seems to participate in several cellular processes, it would be interesting to investigate the involvement of other redox pairs, such as glutathione (GSH) and glutathione disulfide (GSSG), or pyridine nucleotides (NADH and NAD^-) during the morphogenic events in culture. Does the redox state of the cell affect embryo maturation and germination?

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