

## PURINE AND PYRIMIDINE NUCLEOTIDE SYNTHESIS AND DEGRADATION DURING IN VITRO MORPHOGENESIS OF WHITE SPRUCE (*PICEA GLAUCA*)

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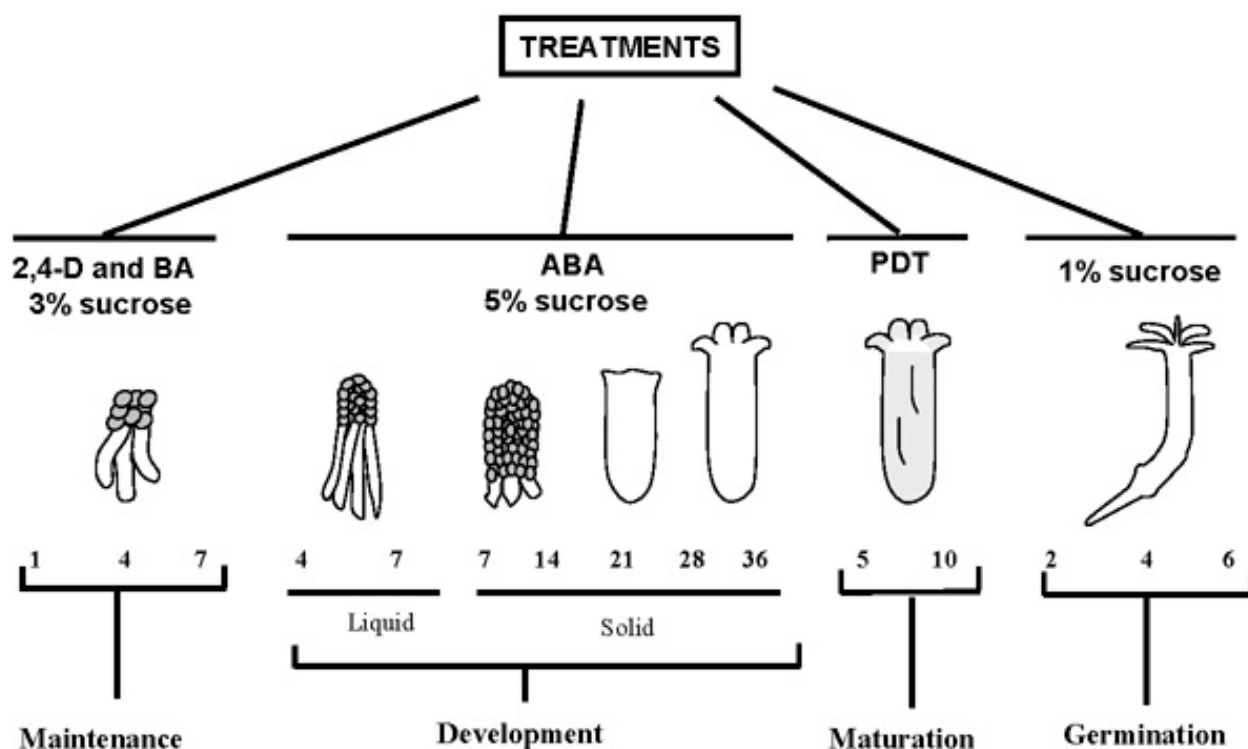
### 1. ABSTRACT

In the last few years, somatic embryogenesis and organogenesis of white spruce (*Picea glauca*) have been used as model systems to investigate biochemical and physiological events related to morphogenesis. This has been possible because studies conducted *in vitro* allow for manipulations of the culture conditions, in which changes in morphogenetic events can be easily related to physiological alterations. De-novo synthesis, salvage, and degradation of both purine and pyrimidine nucleotides are operative at all stages of somatic embryo maturation and germination. Fluctuations in the activity of these pathways delineate important morphogenic events. The early phases of embryo development are accompanied by a decreased salvage activity of purine nucleotides, which reflects a reduction of cell proliferation and the initiation of organized growth. Activities of the salvage enzymes are present throughout the maturation period, and also during the imposition of the drying period, which is required for successful embryo germination. The operative salvage pathway in dried embryos is needed for the enlargement of the nucleotide pool necessary to sustain the reactivation of the overall cellular metabolism at germination, before the reactivation of the de-novo pathway, which is a later event. Manipulations of the culture medium which improve the germination frequency of the embryos also result in increased salvage activity. Similar changes in nucleotide synthesis were also observed during the initiation of shoot development from epicotyl explants of white spruce and

cotyledons of radiata pine. Results from these studies can be used for improving growth and development in culture.

### 2. INTRODUCTION

Purine and pyrimidine nucleotides have numerous functions in plants. They are essential building blocks for nucleic acid synthesis and important components involved in bio-energetic processes. Production of ATP from ADP and phosphate during photosynthesis and respiration is a critical energy conservation step. Similarly, UTP, UDP, and UDP-sugars have a critical role as energy rich precursors during the synthesis of cellulose and matrix components of cell wall (1). In both animal and plant cells, synthesis of several macromolecules, including sucrose, polysaccharides, phospholipids, glycolipids and various secondary products also relies on the availability of nucleotides (2). Furthermore, as a direct link between nucleotide metabolism and plant growth and development, purines are involved in the synthesis of cytokinins (3), important plant growth regulators, and of cyclic monophosphates and ADP-ribose, key components of the intracellular signal transduction pathway (4). Given the involvement of nucleotide metabolism in several cellular processes, it is not surprising that many studies have been generated in the past few decades, including several reviews (1, 2, 5-8). The majority of the topics presented in these reviews, however, deals with nucleotide metabolism in



**Figure 1.** Developmental pathway of white spruce somatic embryos with culture treatments and sampling points. In the maintenance medium the embryogenic tissue consists of early filamentous embryos. Upon subsequent transfers of the tissue into liquid and solid media for development, the embryos increase in size. A well developed shoot and root pole become visible after 21 days on solid medium. During the following days in culture, the embryos develop further, and a ring of cotyledons emerges from the shoot apical region. Fully developed embryos, observed after 36 days on solid medium, are partially dried for 10 days before being transferred on germination medium. Formation of a functional root and shoot can be observed between day 4 and 6. 2, 4-D, 2, 4-dichlorophenoxyacetic acid; BA, N<sup>6</sup>-benzyladenine; ABA, abscisic acid; PDT, partial drying treatment

relation to physiological events occurring during post-embryonic growth. Only little emphasis has been placed on alterations of nucleotide synthesis and utilization during morphogenesis, especially during embryo development and meristem formation. This paucity of information is mainly due to the fact the morphological and physiological studies during embryogenesis and meristem formation are difficult, if not impossible, to carry out *in vivo*. Seed embryos, especially during the early phases of development are embedded in the maternal tissue and therefore difficult to dissect.

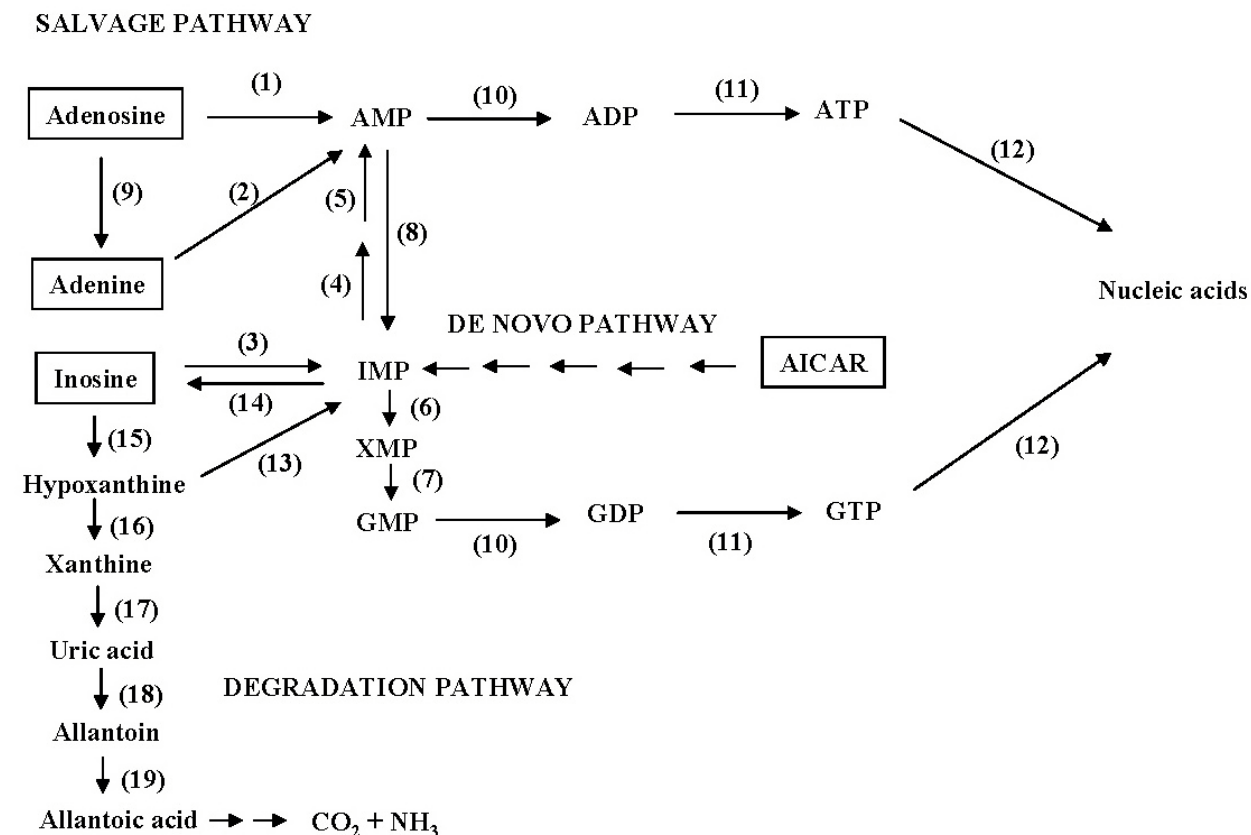
The optimization of tissue culture methods has allowed the generation and propagation of viable plants from somatic cells in culture through two distinct processes: somatic embryogenesis and organogenesis. Compared to somatic embryogenesis, which results in the generation of bipolar structures with a well defined root-shoot axis, organogenesis only produces individual organ types, i.e. shoots or roots (9). Both techniques have been successfully employed in white spruce (10, 11), which is an economically important species in North America, where it is utilized by the forestry industry for wood and lumber production (12). Besides its economic importance, the spruce system is considered by many as the *Arabidopsis* of conifers, since it is an amenable model system to be used

for broad investigations ranging from structure, physiology, biochemistry and molecular biology (13).

The aim of this review is to analyze changes in the de novo synthesis, salvage synthesis, and degradation of purine and pyrimidine nucleotides during somatic embryogenesis and shoot organogenesis in white spruce, and to relate these changes to the morphogenic events associated with both processes.

### 3. PURINE AND PYRIMIDINE METABOLISM DURING SOMATIC EMBRYOGENESIS

Establishment and regeneration of white spruce somatic embryos is successfully achieved through a continuous series of developmental steps, in which a proper execution of each step affects the result of subsequent steps (Figure 1). Therefore, rational manipulations of the culture medium, plant growth regulators, and control of the physical culture environment at each step are critical factors required for successful regeneration of embryos and subsequent growth into normal plantlets (13). In our work, purine and pyrimidine metabolism was investigated during all stages of the process: namely, maintenance of the embryogenic tissue, embryo development, embryo maturation, and germination. Unless otherwise specified,



**Figure 2.** Possible metabolic fate of exogenously supplied adenine, adenosine, inosine, and 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR) during morphogenesis of white spruce. Enzymes: (1) adenosine kinase and/or nucleoside phosphotransferase, (2) adenine phosphoribosyltransferase, (3) inosine kinase, (4) adenylosuccinate synthetase, (5) adenylosuccinase, (6) IMP dehydrogenase, (7) GMP synthetase, (8) AMP deaminase, (9) adenosine nucleosidase, (10) nucleoside monophosphate kinase, (11) nucleoside diphosphate kinase, (12) RNA polymerase, (13) hypoxanthine-guanine phosphoribosyltransferase, (14) 5'-nucleotidase and/or phosphatase, (15) inosine-guanosine nucleosidase, (16) and (17) xanthine dehydrogenase, (18) uricase, (19) allantoinase

synthesis and utilization of purine nucleotides was studied through tracer experiments, by following the metabolic fate of [ $8\text{-}^{14}\text{C}$ ]5-aminoimidazole-4-carboxamide-1- $\alpha$ -D-ribofuranoside (AICAR), intermediate of the de-novo pathway, [ $8\text{-}^{14}\text{C}$ ]adenine and [ $8\text{-}^{14}\text{C}$ ]adenosine, precursors of the salvage pathway, and [ $8\text{-}^{14}\text{C}$ ]inosine, intermediate of the degradation pathway. Additionally, the activity of several enzymes involved in the salvage pathway was also measured (Figure 2). Pyrimidine metabolism was investigated by incubating tissue with [ $6\text{-}^{14}\text{C}$ ]orotic acid, an intermediate of the de novo synthesis, [ $2\text{-}^{14}\text{C}$ ]uridine, a precursor of the salvage pathway, and [ $2\text{-}^{14}\text{C}$ ]uracil, an intermediate of the degradation pathway, as well as measuring the activity of several key enzymes participating in both pathways (Figure 3). Deoxyribonucleotide metabolism was investigated by following the metabolic fate of [ $2\text{-}^{14}\text{C}$ ]cytidine, [ $2\text{-}^{14}\text{C}$ ]deoxycytidine, and [ $2\text{-}^{14}\text{C}$ ]thymidine (Figure 4).

### 3.1. Maintenance of embryogenic tissue

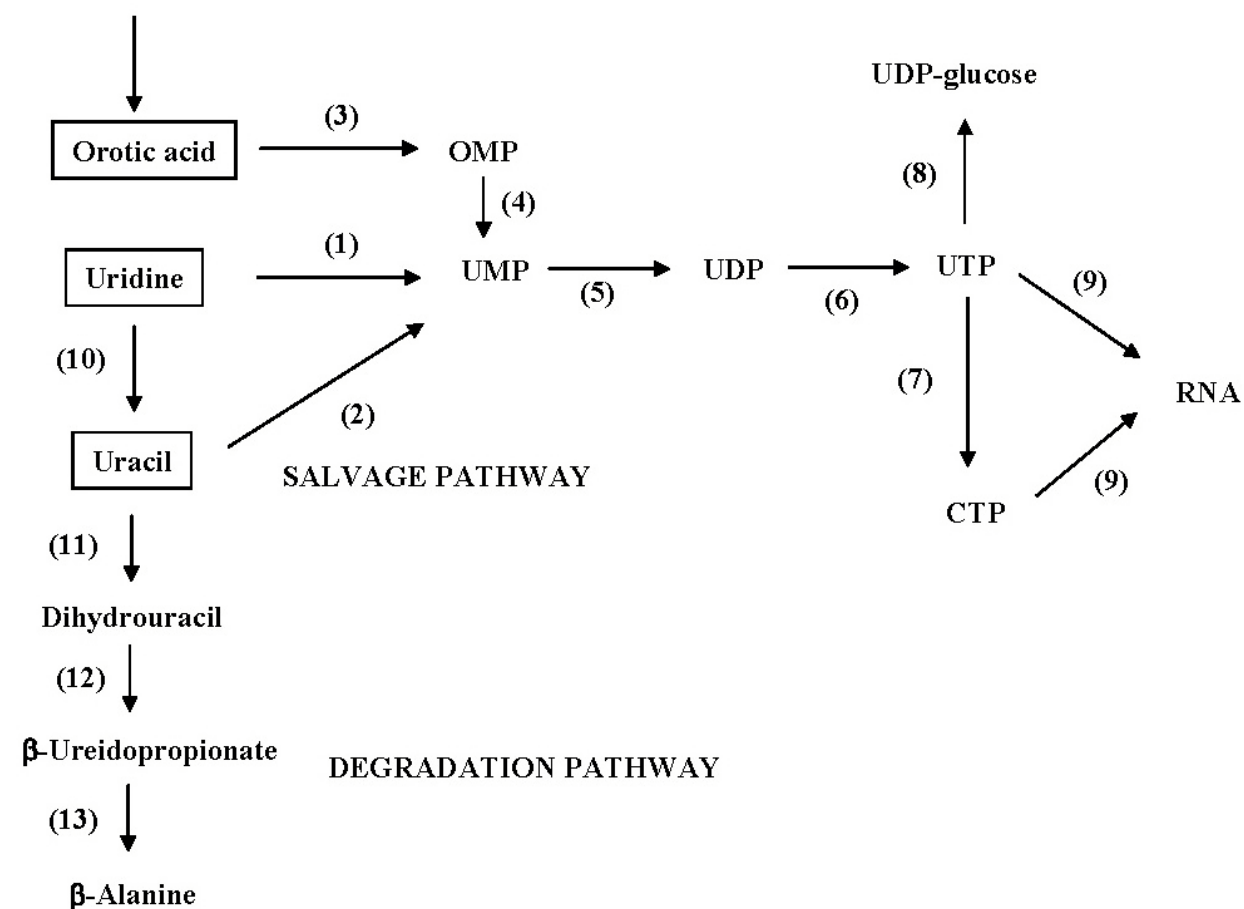
Embryogenic tissue of white spruce, commonly initiated from the hypocotyls region of zygotic embryos (10), appears translucent in color and it is composed of an aggregate of cells from which early filamentous embryos protrude. These immature embryos are characterized by small embryogenic heads, subtended by an elongated

suspensor composed of large, vacuolated cells (13). Proliferation of embryogenic tissue occurs in maintenance media containing 2, 4-dichlorophenoxyacetic acid (2, 4-D) and  $\text{N}^6$ -benzyladenine (BA) (Figure 1). During a typical seven day subculture period embryogenic tissue exhibits a lag phase between day 0 and day 2, an exponential growth phase, between day 2 and 5, followed by a stationary phase from day 5 to day 7 (14).

#### 3.1.1. Purine metabolism during maintenance of embryogenic tissue

Studies on purine metabolism carried out during the three growth phases (at day 1, day 4, and day 7) revealed that both de-novo synthesis, salvage synthesis and degradation pathway are operative during maintenance of white spruce embryogenic cells (14, 15). De novo synthesis of purine nucleotides appears to be active during the all maintenance period. As reported by Belmonte *et al.* (14), radiolabeled AICAR was extensively utilized for nucleotide (AMP+ADP+ATP) and nucleic acid synthesis throughout the maintenance period. In this study it was shown that, although incorporation of AICAR into nucleotides remained constant (36.5, 36, and 31 % of total radioactivity incorporated by cells at day 1, 4, and 7 respectively), incorporation of this intermediate into ADP and ATP was

## DE NOVO PATHWAY



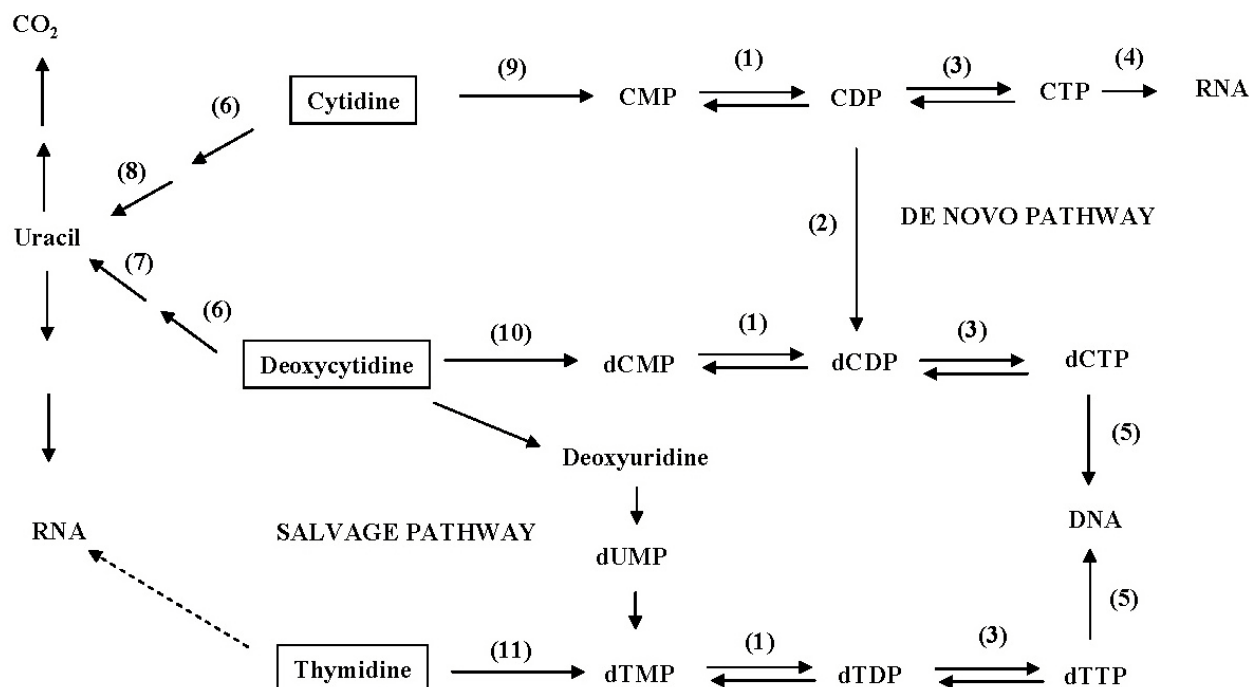
**Figure 3.** Possible metabolic fate of exogenously supplied orotic acid, uridine, and uracil during morphogenesis of white spruce. Enzymes: (1) uridine kinase and/or nucleoside phosphotransferase, (2) uracil phosphoribosyltransferase, (3) orotate phosphoribosyltransferase, (4) orotidine-5'-monophosphate decarboxylase, (5) nucleoside monophosphate kinase, (6) nucleoside diphosphate kinase, (7) CTP synthase, (8) UDP-glucose pyrophosphorylase, (9) RNA polymerase, (10) uridine nucleosidase, (11) uracil reductase and/or dihydrouracil dehydrogenase, (12) dihydropyriminase, (13) ureidopropionase

high during the first 4 days in culture before declining at day 7. This tendency indicates that de novo synthesis of purine nucleotides may be required to increase the endogenous ADP and ATP pool during the exponential growth phase of white spruce cells. The requirement of an active de novo synthesis of purine nucleotides during intense cell division and growth was also demonstrated indirectly by the higher percentage AICAR which was not incorporated into any cellular metabolite at day 7 in culture (31% at day 7 compared to 18% at day 4) (14). Additional comments on the role of the de novo synthesis of purine metabolism during growth and development are difficult to make as very few studies have utilized AICAR as a labeled marker (16).

For the salvage pathway, adenine and adenosine were rapidly taken up by the cells and converted to AMP, possibly by adenosine kinase (AK) and adenine phosphoribosyltransferase (APRT), as very high activities of these two enzymes were detected in gel-filtrated extracts (Table 1). Two routes of adenosine salvage have been demonstrated in plant cells. The first is direct conversion of

adenosine to AMP by adenosine kinase (AK) (or by nucleoside phosphotransferase, NPT(AR)) (step 1, Figure 2), whereas the second involves a sequential conversion of adenosine to AMP via adenine, by the reaction catalyzed by adenosine nucleosidase (step 9, Figure 2) and APRT (step 2, Figure 2). In white spruce embryogenic tissue the first route appears to be preferential, as adenosine nucleosidase activity was never detected at any day in culture and very high levels of AK were measured during the culture period (15). In the maintenance medium the activity of AK was generally higher than that measured for APRT (233, 1330, and 1680 pkat mg<sup>-1</sup> protein for AK and 245, 269, and 289 pkat mg<sup>-1</sup> protein for APRT at day 1, 4, and 7 respectively) (14, 15). In spruce, these differences in enzyme activity are noteworthy, because in the majority of plant tissues investigated, the activity of AK is similar, and often lower, than that measured for APRT (17-19).

In white embryogenic tissue salvage of adenine appears to be mediated by the activity of APRT. Although nucleoside phosphorylase activity was not measured in spruce



**Figure 4.** Possible metabolic fate of exogenously supplied cytidine, deoxycytidine, and thymidine during morphogenesis of white spruce. Enzymes: (1) nucleoside monophosphate kinase, (2) ribonucleotide reductase, (3) nucleoside diphosphate kinase, (4) RNA polymerase, (5) DNA polymerase, (6) cytidine deaminase, (7) thymidine phosphorylase, (8) uridine nucleosidase, (9) cytidine kinase and/or nucleoside phosphotransferase, (10) deoxycytidine kinase and/or nucleoside phosphotransferase, (11) thymidine kinase and/or nucleoside phosphotransferase

cells, salvage of adenine through a secondary route, i.e. conversion of adenine to adenosine catalyzed by adenosine phosphorylase followed by conversion to AMP through activity of AK (see Figure 2), is not likely to occur. The activity of adenosine phosphorylase is generally extremely low in higher plants and in some plants it cannot be detected (6). Studies conducted by Ashihara *et al.* (15) revealed that AMP, produced from the salvage of both adenosine and adenine is rapidly converted to ADP and ATP. However, part of AMP was also converted to guanine nucleotides by sequential reactions catalyzed by AMP deaminase (step 8, Figure 2), IMP dehydrogenase (step 6, Figure 2), and GMP synthetase (step 7, Figure 2). This conclusion was drawn by the authors because some of the radioactivity from both adenine and adenosine was recovered in guanine bases of nucleic acids (15). It is important to note that the active salvage pathway observed in spruce cells may be required for increasing synthesis of nucleotides which are needed as building blocks for nucleic acid synthesis during active cell division. This notion is supported by two independent studies. Firstly, by using different incubation times (2h and 18h) Ashihara *et al.* (15) showed that incorporation of both adenine and adenosine into nucleotides and nucleic acids is a rapid event. After 2h incubation, more than 40% of incorporated precursors, i.e. adenine and adenosine, were recovered into the nucleotide fraction (AMP+ADP+ATP). After 18h, this percentage was reduced to less than 13%, whereas the percentage of radioactivity recovered as nucleic acids (RNA+DNA)

increased from 30% to 50%. Thus a rapid turnover of both adenine and adenosine seems to exist in the maintenance medium. Secondly, a positive correlation seems to exist in white spruce between salvage activity and cell growth. White spruce embryogenic tissue treated with exogenous applications of GSH, which increased the growth of cells possibly by inducing cell division, had a better ability to incorporate radioactivity from both adenine and adenosine into nucleic acids (14). These observations are in agreement with studies conducted in other systems showing the involvement of salvage mechanisms in growth and development (20).

In contrast to adenine and adenosine, utilization of inosine for nucleotide synthesis was very limited in spruce embryogenic tissue (15). This low salvage activity was correlated to the low enzymatic activity of inosine kinase (step 3, Figure 2) and non-specific nucleoside phosphotransferase measured during the seven days in culture (Table 1 and Figure 2). The involvement of these enzymes in inosine anabolism was also documented in a variety of systems, including tobacco (21), black gram (17), and Jerusalem artichoke (22). Although without any direct evidence, Ashihara *et al.* (15) speculated that an alternative metabolic route for inosine in spruce cells may involve an initial conversion to hypoxanthine by inosine-guanosine nucleosidase (step 15, Figure 2), followed by salvage to IMP by hypoxanthine-guanine phosphoribosyltransferase (step 13, Figure 2). The activities of both enzymes, in fact have been demonstrated in several other plant species (17, 18, 23-25).

**Table 1.** Enzymatic activity of the major enzymes of purine and pyrimidine metabolism during maintenance, development, maturation, and germination of white spruce somatic embryos

	Maintenance			Development							Maturation		Germination		
	1	4	7	Liquid medium		Solid medium					5	10	2	4	6
PURINES															
AK	233	1130	1680	968	566	1705	978	866	845	156	114	148	153	232	212
IK	12	16	13	21	14	22	28	18	29	4	1	ND	2	3	2
APRT	245	269	289	432	487	476	732	628	614	153	172	200	210	591	593
NPT(AR)	16	13	11	12	17	15	14	14	19	2	4	3	3	3	2
NPT(IR)	9	3	4	12	9	16	15	15	19	2	2	2	2	3	2
ARN	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	5	5
PYRIMIDINES															
UK	120	63	57	40	26	72	75	53	55	50	31	46	13	23	6
UPRT	10	20	2	10	3	5	7	5	24	3	2	2	1	2	3
OPRT	22	157	172	130	143	151	60	72	63	23	23	32	21	37	39
PRPP(s)	-	75	30	91	63	57	105	97	107	16	31	32	22	40	27
NPT(UR)	9	13	10	7	8	6	7	8	16	1	2	1	1	1	1

Values are expressed as pkat mg<sup>-1</sup> protein. AK, adenosine kinase; IK, inosine kinase; APRT, Adenine phosphoribosyltransferase; NPT, nucleoside phosphotransferases measured with adenosine (AR), inosine (IR), and uridine (UR); ARN, adenosine nucleosidase; UK, uridine kinase, UPRT, uracil phosphoribosyltransferase, OPRT, orotate phosphoribosyltransferase; PRPPs, 5-phosphoribosyl-1-pyrophosphate synthase. ND, not detected; -, not measured

Degradation of inosine by white spruce cells occurs very rapidly. Throughout the maintenance period, a substantial fraction of radioactivity recovered from labeled inosine was found as allantoic acid (66%) and CO<sub>2</sub> (66%) after 2h incubation. After 18h incubation period almost all inosine (more than 95%) was released as CO<sub>2</sub> (15). An active degradation pathway, estimated by the metabolic fate of exogenously supplied inosine was also documented in other plant species (26).

### 3.1.2. Pyrimidine metabolism during maintenance of embryogenic tissue

An active de novo synthesis was documented in white spruce cells during the maintenance period. Ashihara *et al.* (15) showed that [6-<sup>14</sup>C]orotic was efficiently utilized for the synthesis of uridine nucleotides and UDP-glucose. In this study it was demonstrated that more than 56% of incorporated orotic acids was recovered in these fractions after a short (2h) incubation period. After longer incubation times (18h) this percentage decreased significantly, whereas that recovered as nucleic acids increased sharply (to more than 40%). These results, which were interpreted by the authors as a rapid turnover of orotic acid (i.e. conversion of to nucleotides followed by incorporation into nucleic acids) were possibly due to the activity of orotate phosphoribosyltransferase (OPRT) (step 3, Figure 3), which was always measurable throughout the course of the experiment (14, 15). Of interest, the activity of this enzyme increased significantly from the lag phase (day 1) to the following exponential growth phase (day 4) and remained high during the stationary phase (day 7) (Table 1). This trend, suggests that activation of this enzyme may be related to active cell division and growth of spruce cells. The activity of 5-phosphoribosyl-1-pyrophosphate synthase (PRPPs), another enzyme of the pyrimidine de novo synthesis which catalyzes the formation of 5-phosphoribosyl-1-pyrophosphate from ribose-5-phosphate

and ATP was also measured at day 4 and 7 of development (15). The higher activity of PRPPs during the exponential growth of spruce cells (day 4) compared to the stationary phase (day 7) (Table 1) also confirms the importance of the de novo pathway of pyrimidine metabolism during active cell proliferation. The active utilization of orotic acid for nucleotide synthesis observed in spruce is in agreement with previous work (27, 28), which demonstrates the operation of the pyrimidine de novo pathway during plant growth.

Studies on the salvage pathway indicate that uridine is actively salvaged to nucleotides and nucleic acid during the maintenance period (14, 15). In these studies it was demonstrated that more than 70% of the radioactivity from uridine was salvaged after only 2h incubation. Also, as observed for orotic acid, a rapid turnover of this precursor existed in white spruce cells. A large proportion of uridine taken up by the cells is first converted to nucleotides, after just 2h incubation, and then rapidly incorporated into nucleotides (15). This efficient salvage activity was ascribed to the high activity of uridine kinase (and to a lesser extent to the activity of non-specific phosphotransferases, NPT(UR)) (step 1, Figure 3) (Table 1). The alternative route of uridine salvage, i.e. conversion to uracil by uridine nucleosidase (step 10, Figure 3) followed by conversion to UMP by uracil phosphoribosyltransferase (UPRT) (step2, Figure 3), appears to be less operative. The low activity of UPRT compared to UK (Table 1) and the limited amount of radioactivity from uridine recovered as uracil (15) support this notion. Compared to orotic acid, however, utilization of uridine for nucleotide synthesis is less efficient in spruce cells. This result, which is in agreement with studies conducted in jack pine (27), is different from data obtained from suspension-cultured *C. roseus* cells (29) and carrot cells (28). In these two systems, in fact, uridine was the

best precursor for nucleotide and nucleic acid synthesis. Based on these observations, it appears that differences in utilization of pyrimidine precursors may exist between gymnosperms and flowering plants.

The degradation pathway of pyrimidine metabolism is also operative in spruce embryogenic tissue, as demonstrated by the rapid catabolism of radiolabeled uracil. Two independent studies (15; Belmonte, Loukanina, Stasolla, Yeung, and Thorpe, unpublished results) demonstrated that after only 2h incubation, more than 70% of the radioactivity from uracil was recovered as CO<sub>2</sub>, and a lower percentage as  $\beta$ -ureidopropionate. Based on these observations it was suggested that the pathway of uracil degradation involves an initial conversion of uracil to dihydrouracil (step 11, Figure 3), followed by a series of reaction leading to the production of  $\beta$ -alanine, NH<sub>3</sub> and CO<sub>2</sub> (15). The observed degradation of uracil in white spruce cells is mainly ascribed to the low activity of UPRT (Table 1). A correlation between rate of uracil degradation and activity of UPRT (step 2, Figure 3) was also observed in other systems (28-31), thus denoting similar metabolic regulation among plant species. In spruce, however, UPRT activity itself may not be the rate limiting step for uracil utilization. As reported by Ashihara *et al.* (15), the activity of this enzyme is 10 times higher in day 4 cells than in day 7 cells (Table 1), although the rate of uracil utilization (incorporation into nucleotides and nucleic acids) did not change between the two days. This observation is agreement with previous studies conducted on tobacco leaves (28).

### 3.2. Development of somatic embryos

Induction of embryo development in white spruce is initiated by transferring embryogenic tissue into a liquid development medium, containing 5% sucrose and abscisic acid (ABA) as a sole exogenous plant growth regulator, for 7 days. During this period, proliferation of the tissue is reduced and filamentous embryos develop further. The embryogenic heads of the embryos increase in size and the suspensor region becomes more structurally organized (Figure 1). A liquid environment, however, is not suitable for continuation of embryo development, and tissue must be transferred for 36 days onto solid medium. During development, somatic embryos undergo a series of dramatic morphological and physiological changes which include the formation of functional shoot and root apical meristems, the elongation of the hypocotyl axes, and the inception of a ring of cotyledons from the apical notch of the embryos (32) (Figure 1).

#### 3.2.1. Purine metabolism during development of somatic embryos

Studies on purine salvage during white spruce somatic embryo development revealed that both adenine and adenosine were easily salvaged and utilized for ATP and nucleic acid synthesis during all stages of embryo development (33). In the same study it was shown that the metabolic fate of both labeled precursors was very similar throughout development. After a short incubation time (2h) the nucleotide fraction (mainly ATP) was more labeled than the nucleic acid fraction, but after 18h the opposite

was observed. This observation is indicative of a rapid turnover of both adenine and adenosine as the embryos developed in culture. It is important to note that transfer of embryogenic tissue from the maintenance medium to the development medium is associated with a small, but consistent decrease of salvage activity. Specifically, the incorporation of both adenine and adenosine into the nucleotide fraction, expressed as a percentage of total incorporation, decreased from more than 50% in the maintenance medium to less than 40% at day 4 in the ABA-containing liquid medium (Figure 1) (33). If an active salvage pathway during the maintenance medium may be required for the enlargement of the endogenous nucleotide pool, necessary for rapid cell proliferation, a reduction of the salvage activity at the beginning of embryo development may be needed for a reduction of cell proliferation and initiation of organized growth of the embryos. It has been speculated that these alterations in salvage activity may represent a metabolic switch which terminates proliferation and initiate embryonic development (13). If proven to be true, this notion opens new possibilities of improving the yield of embryos produced in culture through experimental manipulations of the salvage pathway of purines. Another plausible interpretation of the data is that a reduction of salvage activity, observed at the onset of embryo development, may not be a true reflection of the actual rate of purine salvage in different tissue types of the embryo. With the formation of new tissues, in fact, it cannot be excluded that high salvage activity may still be present, but restricted to specific regions or tissues of the developing embryos where cell division is occurring. Tissue-dependent studies on metabolic processes are difficult, if not impossible, to carry out in embryos, which are per se very small and difficult to manipulate. Investigations conducted by Ashihara *et al.* (33) also showed that if a reduction in adenosine salvage at the inception of embryo development can be related to a reduction in AK activity (step 1, Figure 2), the reduced utilization of adenine does not reflect a decline in APRT activity (step 2, Figure 2). Thus it is possible that mechanisms other than enzymatic activity may control the metabolic fate of this precursor in developing embryos.

As embryo development progresses on solid medium, utilization of both adenine and adenosine for synthesis of salvage products slowly declines. This decline is likely due to a reduction in the activities of both APRT and AK (Table 1). Among the possible reasons behind this tendency is the formation of organelles in developing somatic embryos. Since all major salvage enzymes, including AK and APRT are localized in mitochondria and plastids, as well as in the cytosol (34, 35), it is plausible to assume that the developmental control of the number of these organelles during embryo growth may regulate the activity of the salvage enzymes, thus affecting the rate of purine salvage.

Compared to adenine and adenosine, only a limited amount of radioactivity from labeled inosine was incorporated into salvage products, i.e. nucleotides and nucleic acid, throughout embryo development. A large fraction of this precursor was degraded as ureides and CO<sub>2</sub>



and this was mainly due to the low activity of IK (step 3, Figure 2), the enzyme responsible for the salvage of inosine (Table 1). Although there is no information on the activity of enzymes involved in purine catabolism during embryo development in white spruce, results from tracer experiments indicate that inosine nucleosidase, xanthine dehydrogenase, and uricase (steps 15, 17, 18, Figure 2) are very active during embryo growth (33).

### 3.2.2. Pyrimidine metabolism during development of somatic embryos

The de novo pathway of pyrimidine metabolism was very active throughout embryo development. After only 2h incubation, most radioactivity (59-72%) from orotic acid taken up by the developing embryos was incorporated into nucleotides, and more than 13% of total radioactivity was recovered as nucleic acids (36). Thus more than 70% of supplied orotic acid was quickly utilized for nucleotides and nucleic acid at all stages of development. As observed during the maintenance period, turnover of orotic acid is very fast in developing embryos. This observation is not surprising since intense nucleotide synthesis must occur during development to sustain the growth of the embryos. The high activity of OPRT (step 3, Figure 3), especially during the initial phases of embryo development (Table 1), may account for the active utilization of orotate and for its limited catabolism. As reported by Ashihara *et al.* (36), in fact, less than 3% and 24% of radioactivity from orotic acid was released as CO<sub>2</sub> after 2h and 18h incubation. The rapid anabolism of orotic acid during embryogenesis appears to be a common feature across species, as a similar result was reported in embryogenic cells of carrot (28).

Similar to the de novo pathway, salvage of pyrimidines, estimated by the utilization of uridine for nucleotides and nucleic acid synthesis, was extremely active during spruce embryo development. As documented by Ashihara *et al.* (36), more than 50% and 20% of total radioactivity from labeled uridine was recovered as nucleotides after 2h and 18h incubation time. As also observed for the de novo pathway, turnover of uridine was extremely fast throughout the developmental period, as exogenously supplied uridine was converted first into nucleotides and then incorporated into the nucleic acid fraction (see Figure 3). The activity of UK (step 1, Figure 3) which remained high during embryo development (Figure 1) was responsible for the large utilization of uridine for the synthesis of salvage products (36) (Table 1).

In developing embryos of white spruce a large fraction of uracil (more than 70%) taken up by the embryos is catabolized to CO<sub>2</sub> and β--ureidopropionate (36). Active degradation of pyrimidine nucleotides was found to be constant throughout development and was related to the low activity of the uracil salvage enzyme, UPRT (step 2, Figure 3). The activity of this enzyme, in fact, was always much lower than that of UK (step 1, Figure 3) during the growth of the embryos (Table 1) (36).

Despite the limited information available on the biosynthesis of deoxyribonucleotides in plants, the

existence of the de novo and salvage pathways has been demonstrated recently in developing white spruce somatic embryos by following the metabolic fate of [2-<sup>14</sup>C]cytidine, [2-<sup>14</sup>C]deoxycytidine, and [2-<sup>14</sup>C]thymidine (Figure 4) (37). The de novo synthesis of deoxyribonucleotides appears to be operative during embryo development since a low, but significant amount of radioactivity from cytidine is recovered in DNA. As reported by Stasolla *et al.* (37), in fact, a proportion of supplied cytidine is converted to nucleotides after 2h incubation, and then incorporated into DNA. Besides its utilization for DNA synthesis, developing embryos of spruce have the ability to incorporate cytidine into RNA. This conversion can occur directly, after conversion to CMP, CDP, and CTP (steps 1-4, Figure 4), or indirectly, via uracil (steps 6, 8, Figure 3). Direct conversion seems to be regulated by cytidine kinase (step 9, Figure 4). The activity of this enzyme, detected in several plant species (38-40), has a similar profile to that observed for the incorporation of cytidine into RNA throughout development. Specifically, both activity of cytidine kinase and utilization of cytidine for RNA synthesis increased as the embryos developed (37). These tendencies may be associated with the many physiological changes occurring during embryo growth. Although not demonstrated, indirect incorporation of cytidine into RNA, via deamination to uridine and ammonia by cytidine deaminase activity (step 6, Figure 3) (40, 41), may also be operative in spruce cells. Stasolla *et al.* (37) demonstrated that in developing embryos of spruce labels from [2-<sup>14</sup>C]cytidine was recovered as uracil and uridine. This indirect route of cytidine incorporation has been demonstrated in bean leaves (42).

Tracer experiments conducted with labeled thymidine and deoxycytidine also indicate that the salvage synthesis of deoxyribonucleotides is active during development of spruce embryos (Figure 4). Both precursors, in fact, are rapidly metabolized and incorporated into nucleotides and nucleic acids (37). Although observed during development, salvage of thymidine was very limited, as a large percentage of radioactivity from this precursor was recovered as degradation products, mainly CO<sub>2</sub>. Of interest, salvage of thymidine increased during embryo development and this was ascribed to the increased activity of thymidine kinase (step 11, Figure 4) (37). Although not detected in several systems (43, 44), the activity of this salvage enzyme is strictly correlated with the rate of DNA synthesis. In synchronized animal cells, for example, thymidine kinase activity was low outside the S phase of the cell cycle, but reached highest levels at the beginning of DNA replication (45).

In contrast to thymidine, utilization of deoxycytidine for nucleic acid and nucleotide synthesis (Figure 4) is very active at all stages of embryo development. Turnover of this precursor is extremely fast as a large proportion of radioactivity from [2-<sup>14</sup>C]deoxycytidine is first recovered as nucleotides after 2h incubation and then utilized for nucleic acid synthesis after 18h (37). Incorporation of deoxycytidine into the DNA fraction is mediated by the salvage enzyme deoxycytidine



kinase (step 10, Figure 4). The activity of this enzyme has been demonstrated in spruce where it remains high throughout the developmental period (37). As observed for cytidine, a fraction of radioactivity from deoxycytidine was recovered in RNA, possibly after conversion to uracil, catalyzed by cytidine deaminase and thymidine phosphorylase (steps 6 and 7, Figure 4). The recovery of label from deoxycytidine into uracil, uridine, and deoxyuridine, as well as in uracil bases from hydrolysis of RNA confirms this route (37).

### 3.3. Maturation of somatic embryos

Fully developed somatic embryos of white spruce cannot germinate properly unless they undergo a maturation period. Methods for maturing somatic embryos in conifers have been described in several reviews (32), and all result in a decrease of the embryonic moisture content. In white spruce, a common method of maturation is the partial drying treatment (PDT) (46), in which fully developed embryos are placed in the central wells of tissue culture plates with the outer wells full with sterile water for 10 days. During the PDT several physiological and biochemical changes occur and they seem to be required to terminate the developmental program in preparation for subsequent germination. They include changes in storage product deposition pattern and alterations in endogenous content of abscisic acid and ethylene (32). Partial dried embryos of white spruce have higher germinability and improved post-embryonic growth compared to embryo germinated without a prior imposition of a PDT.

#### 3.3.1. Purine metabolism during maturation of somatic embryos

Both the purine salvage and degradation pathways were found to be operative during the 10 days of the partial drying treatment (Figure 2). Although the amount of radioactivity from both adenine and adenosine recovered as salvage products is lower compared to that observed during embryo development, a fast turnover of both precursors was observed in drying embryos (47). A significant proportion of adenine and adenosine, in fact, is converted into nucleotides after 2h incubation and nucleic acid after longer (18h) incubation time. The operative purine salvage, together with the increased specific activity of APRT (Table 1; step 2, Figure 2) observed during maturation, may be required for the enlargement of the nucleotide pool necessary to sustain the reactivation of the overall cellular metabolism at germination. The participation of the purine salvage pathway to the enlargement of the nucleotide pool at the inception of germination has been well documented in literature (17, 20, 24). These results suggest that poor post-embryonic growth observed in embryos germinated without the imposition of a PDT may be in part ascribed to the inability of these embryos to generate purine nucleotides through the salvage activity.

During embryo maturation, a large fraction of supplied inosine is degraded to CO<sub>2</sub> and ureides, thus indicating the presence of an active degradation pathway of purine metabolism. As indicated previously, limited utilization of inosine for nucleotide synthesis is due to the

low activity of IK (step 3, Figure 2), which remained low though the PDT (Table 1) (47). It is worth noting, however, that as the embryos dry, the proportion of radioactivity from inosine recovered into the ureide fraction increased. Ureides, which were found to be the major product of purine catabolism in cotyledons and embryonic axes of black gram seedlings (17), may constitute a source of energy to be utilized at the inception of germination. The role of ureides, i.e. allantoin and allantoic acid (Figure 2), as a nitrogen source is well documented (48).

#### 3.3.2. Pyrimidine metabolism during maturation of somatic embryos

An active *de novo* synthesis of pyrimidine nucleotides (Figure 3) was observed during the PDT of spruce somatic embryos. Supplied orotic acid, in fact, was converted rapidly into nucleotides and nucleic acid at both day 5 and 10 of the PDT (47). Active utilization of this precursor was not observed in dry black gram seeds (30). This was associated with a low content of 5-phosphoribosyl-1-pyrophosphate, an important substrate of the pyrimidine *de novo* pathway (49). This discrepancy in results can be explained by the limited water loss experienced by partially dried somatic embryos, compared to fully desiccated seeds. Although the endogenous content of 5-phosphoribosyl-1-pyrophosphate was not measured in white spruce, it can be argued that this metabolite is present in partially dried white spruce somatic embryos, as the activity of PRPPs, the enzyme that which catalyzes the formation of 5-phosphoribosyl-1-pyrophosphate from ribose-5-phosphate and ATP, increased during the PDT (Table 1). The increased activity of this enzyme, which was also observed during the lag phase of periwinkle cells (50), may be required for the *de novo* biosynthesis of pyrimidine nucleotides needed to support cell division at germination.

Partial dried embryos are also able to synthesize nucleotides via the salvage mechanism (Figure 3). More than 75% of the incorporated uridine was recovered as nucleotides and nucleic acids at both day 5 and 10 in PDT. This active salvage mechanism is due to the activity of UK (step 1, Figure 3), which was found to increase slightly during the maturation period (Table 1) (47). The presence of UK activity, also found in dry wheat embryos (51) and in cultured cells during the lag phase (50), indicates that that PDT may be required for the enrichment of the uridine nucleotide pool during the early phases of germination.

Compared to uridine a large fraction of exogenously supplied uracil is degraded to CO<sub>2</sub> and  $\beta$ -ureidopropionate (Figure 3). This tendency was observed at both day 5 and day 10 during the maturation period and was due to the low activity of the major uracil salvage enzyme: UPRT (step 2, Figure 3) (Table 1) (47).

### 3.4. Germination of somatic embryos

Germination of somatic embryos is carried out by placing partially dried somatic embryos on a solid medium with a low sucrose concentration (1%) and devoid of plant growth regulators. Resumption of cell division is observed after a few days in culture, and it is soon followed by the reactivation of the root and shoot apical meristems, which

contribute to the emergence of a functional root and shoot (Figure 1) (52). For more information on the morphological and physiological events occurring during germination of white spruce somatic embryos please refer to Stasolla and Yeung (13).

### 3.4.1. Purine metabolism during germination of somatic embryos

In white spruce, as in other species (20) the metabolic pattern of purine metabolism during germination can be divided in three distinct phases: (1) an unmetabolized phase, (2) a salvage phase, and (3) ureide formation phase. During the early stages of germination, the total uptake of purine precursors, as well as their incorporation into the different cellular fractions is low. A significant fraction of all purine precursors, in fact, remained unmetabolized (53). Such results may be due to the absence of full tissue hydration during the first hours on the germination medium; therefore, the whole machinery involved in the utilization of purine precursors is probably not fully functional.

The second phase of purine metabolism is the “salvage phase”, in which both adenine and adenosine are actively utilized for nucleotide and nucleic acid synthesis (Figure 2). After 2h incubation, utilization of adenine for the synthesis of nucleic acids increased from less than 20% at day 2 to more than 70% at day 6. Similar, but less pronounced changes were also observed for adenosine (53). Salvage of adenine during germination appears to be regulated by the activity of APRT (step 2, Figure 2), which increased significantly from day 2 to day 6 (Table 1), whereas two different mechanisms control the salvage of adenosine. The first one is the single step reaction catalyzed by AK (step 1, Figure 2). The activity of this AK, which was described as the only enzyme responsible for the salvage of adenosine during maintenance of embryogenic tissue, development, and maturation of somatic embryos almost doubled during the first six days of germination (Table 1). The second route of adenosine salvage, involves the hydrolysis of adenosine to adenine by adenosine nucleosidases (ARN step 9, Figure 2), followed by the addition of a phosphoribosyl group catalyzed by APRT (step 2, Figure 2). This latter salvage mechanism, not active during the previous steps of somatic embryogenesis, appears to be operative after day 4 in the germination medium, as denoted by the activity of ARN (Table 1) (53). Adenosine nucleosidase, which is the preferential enzyme of adenosine salvage in leaves of peach trees (54), seems to be strictly associated to germination. Similarly to the results obtained in white spruce, in fact, ARN was found to be absent in dried *Lupinus luteus* L. seeds, but increased significantly following imbibition (55). The extensive utilization of adenine and adenosine for the synthesis of salvage products seems to be critical for successful germination of white spruce somatic embryos. Previous studies have revealed that applications of ascorbic acid, which increase the germination frequency of the somatic embryos through the reactivation of meristematic activity (56), also result in increased salvage of both adenine and adenosine (53). These studies open new possibilities for improving the post-embryonic performances of the somatic

embryos through experimental manipulations of the salvage pathway of purine metabolism.

The third phase of the metabolic pattern of purine metabolism, following the “salvage phase”, is the formation of ureides, which represent an important nitrogen source during the early phases of germination, particularly in leguminous species (48). As suggested by Fujiwara and Yamaguchi (57), the main pathway for allantoin and allantoic acid formation is the degradation of purines (Figure 2). This concept appears to hold true in germinating spruce somatic embryos as a conspicuous formation of ureides derived from degradation of supplied adenine, adenosine, and inosine was observed after six days in germination (53).

### 3.4.2. Pyrimidine metabolism during germination of somatic embryos

As observed with purine nucleotide metabolism, changes in pyrimidine metabolism delineate important stages of embryo germination. Although both *de novo* and salvage pathways are operative in germinating embryos of white spruce, their contribution to the enlargement of the nucleotide pool during the process appears to be differentially regulated. Metabolism of pyrimidines during germination can be divided in two separate phases: a “salvage phase”, at the inception of germination and a “*de novo* phase”, observed at later stages (30, 58). In spruce somatic embryos, for example, uridine utilization for nucleotide and nucleic acid synthesis is high during the early phases of germination and it slowly declines during the following days, possibly due to a reduction of the activity of UK (Table 1) (58). This pattern does not appear to be unique to *in vitro* conditions, as preferential incorporation of uridine into nucleic acid synthesis was also observed during the early phases of germination in zygotic spruce embryos (59). The predominant role of the salvage pathway at the inception of germination may represent a strategy necessary for supplying embryos with sufficient nucleotides before the reactivation of the *de-novo* machinery, which requires some time before becoming fully operative. Utilization of orotic acid for synthesis of nucleotides and nucleic acids via the *de-novo* pathway is in fact low in mature embryos and slowly increases as germination progresses. The reactivation of *de novo* synthesis closely matches the activity of OPRT (step 3, Figure 3), which also increases in germinating embryos (Table 1) (59). Similar changes in OPRT activity were also reported in germinating seeds of other species (49, 60). Although not determined in spruce, the activities of other enzymes participating in the construction of the pyrimidine ring, including carbamoyl phosphate synthase and aspartate carbamoyltransferase, have been found to increase during the later stages of germination (51). These data reinforce the concept that the *de novo* machinery of pyrimidine nucleotides is not fully activated at the onset of germination. During these early phases the salvage pathway is the major contributor to the synthesis of nucleotides.

Studies on deoxyribonucleotide metabolism also revealed that both *de novo* and salvage pathways are operative during germination of white spruce embryos

(Figure 4). The activity of the de novo pathway was demonstrated by the low, but significant utilization of cytidine for DNA synthesis. Conversion of this precursor to CMP, was mainly regulated by the activity of cytidine kinase, and to a lesser extent by non-specific phosphotransferases (step 9, Figure 4) (37). The involvement of cytidine kinase during the early phases of germination has also been documented by Wanka and Bauer (38).

Similarly to the de novo pathway, salvage synthesis of deoxyribonucleotides was active during germination. In germinating white spruce somatic embryos, the utilization of deoxycytidine for DNA synthesis was higher than that measured in fully matured embryos (37). In the same study it was shown that both deoxycytidine kinase and non-specific phosphotransferases contributed equally to the salvage mechanism. Compared to deoxycytidine, a large proportion of exogenously supplied thymidine was degraded to CO<sub>2</sub> during the early phases of germination (37). This tendency was also demonstrated in germinating black gram seeds (44). As germination progresses, however, spruce embryos acquire a better ability to utilize thymidine for nucleic acid synthesis (37). In white spruce, thymidine metabolism, especially the salvage pathway, appears to be very important for successful germination. Manipulations of the culture environment that decrease the germination frequency of the embryos also increase the rate of thymidine degradation and decrease the salvage activity (Stasolla and Yeung, unpublished data). Anabolism of thymidine in spruce is mediated by the activity of thymidine kinase, as well as non-specific phosphotransferases (step 11, Figure 4). The contribution of these two enzymes to the salvage of thymidine appears to be species dependent. The activity of thymidine kinase, for example, was not detected during germination of black gram seed (44). In this system, non-specific phosphotransferase activity was the only enzyme responsible for the conversion of thymidine to TMP. In somatic and zygotic embryos of spruce, on the other hand, both enzymes seem to contribute equally to the salvage of thymidine (37, 59).

#### 4. PURINE AND PYRIMIDINE METABOLISM DURING ORGANOGENESIS

To date, only two unpublished studies dealing with purine and pyrimidine metabolism during shoot organogenesis are available, and they have been conducted on white spruce and radiata pine (Stasolla, Loukanina, Ashihara, Yeung, and Thorpe, unpublished data). Studies during shoot formation from epicotyls of white spruce revealed that production of purine nucleotides via the salvage pathway plays an important role during the process. Specifically, epicotyls cultured under shoot-forming conditions are able to better incorporate both adenine and adenosine into nucleotides and nucleic acids. This ability, which was reduced in epicotyls cultured on non-shoot forming medium, was due to the increased high activity of APRT and AK (steps 2 and 1 resp., Figure 2) after 10 days in culture. Although not demonstrated in spruce, reactivation of the salvage pathway of pyrimidines may

also be important during the process. The early phases of shoot organogenesis from cotyledons of radiata pine, for example, are characterized by increased incorporation of uracil into the nucleic acid fraction and a higher activity of UPRT (Stasolla, Loukanina, Ashihara, Yeung, and Thorpe, unpublished results). Therefore, as indicated for germinating embryos of spruce, the activation of the salvage pathway during shoot organogenesis may represent a metabolic switch which initiates the morphogenic events leading to shoot formation.

#### 5. CONCLUSIONS

Studies conducted during the *in vitro* morphogenesis of white spruce have been valuable in establishing the pattern of purine and pyrimidine metabolism during growth and organized development in culture. Besides confirming concepts already established in the literature, our work on somatic embryogenesis and organogenesis has established the role played by purine and pyrimidine in relation to morphogenesis. This has been possible because studies conducted *in vitro* allow for manipulations of the culture conditions in which changes of morphogenetic events can be easily related to alterations of metabolism. The most important picture emerging from our work is that the contribution of the salvage pathways to both purine and pyrimidine nucleotide biosynthesis play a critical role for completion of growth and development in culture. Many events in culture, including the initiation of embryo development, the early phases of embryo germination and shoot formation required active salvage activity. Besides its theoretical implications, this information can be used for further studies aimed at improving growth in culture.

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