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Seed Germination in *Larix decidua*

by

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With 12 Figures

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Summary

TREVISAN R., MARIANI P., RASCIO N., VENTURINI M. G. & BALDAN B. 1990. Seed germination in *Larix decidua*. – Phyton (Horn, Austria) 30 (1): 1–13, 12 figures. – English with German summary.

The germination of *Larix decidua* seeds and the achievement of the seedling photosynthetic competence were examined considering both ultrastructural and physiological aspects. The distribution of the reserves as well as the rate of their mobilization were compared in both gametophytic (endosperm) and sporophytic (embryo) tissues. A short period of cold acclimatization enhanced the rate of germination and of endosperm reserve utilization. Cotyledons acquired the photosynthetic activity early during germination, as shown by the oxygen release values and the ultrastructural features of the chloroplasts.

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Zusammenfassung

TREVISAN R., MARIANI P., RASCIO N., VENTURINI M. G. & BALDAN B. 1990. Samenkeimung bei *Larix decidua*. – Phyton (Horn, Austria) 30 (1): 1–13, 12 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Die Keimung von *Larix decidua*-Samen und das Erreichen der Fähigkeit der Sämlinge zur Photosynthese werden in ultrastruktureller und physiologischer Hinsicht untersucht. Die Verteilung der Reservestoffe im Gametophyten (Endosperm) und im Sporphyten (Embryo) und ihre Mobilisation werden verglichen. Eine kurze Kälteperiode steigerte die Keimungsrate und den Aufbrauch der Reserven im Endosperm. Sauerstoffausscheidung sowie ultrastrukturelle Merkmale der Chloroplasten lassen den Eintritt photosynthetischer Fähigkeit der Kotyledonen in frühen Keimungsstadien erkennen.

Introduction

Embryo and young seedlings are used for propagation in vitro of several Conifer species (BONGA 1988, FOWKE & HAKMAN 1988, NARAYANASWAMY 1988). In particular, several reasons make larches suitable for species improvement through micropropagation (DINER & al. 1986). Basic information on germination at cytological and physiological level can contribute to correlate organogenic competence with developmental stages of the seedlings (see, for example, AITKEN-CHRISTIE & al. 1985). Unlike für Angiosperm seeds (BEWLEY & BLACK 1983), knowledge of germination of Conifer seeds is scanty. Ultrastructural changes in germinating seeds have been previously reported by DURZAN & al. 1971, SIMOLA 1974 a, b, 1976, DE CARLI & al. 1987 for two *Pinus* species and *Picea abies*. As far as we know, cytological studies on *Larix decidua* seed germination are lacking. In the present paper we report ultrastructural changes in both embryo and gametophyte tissues of *Larix decidua* seeds during germination. The development of the cotyledon photosynthetic activity was also assessed through oxygen release and chlorophyll content measurement.

Materials and Methods

Larix decidua Miller seeds were supplied by the Centro Produzione Sementi Forestali, Ministero Agricoltura e Foreste, Peri (Verona, Italy) and came from Cavedine (TN). After a rapid surface-sterilization with 5% Na hypoclorite, the seeds were imbibed in tap water for 15 h, placed on moist filter paper in Petri dishes and then grown in a growth chamber with a 12 h thermoperiod (20°C and 27°C) and 12 h photoperiod with light of 4500 lux at ground level, produced by Philips TLMF 40 fluorescent tubes.

A set of seeds was stratified on sand at 4°C in the dark for 15 days and then sown as above. The sowing was considered as the begin of the experiments.

Specimens were sampled from embryo and endosperm of dry and imbibed seeds and from seedlings every two days. Since germination was not uniform, after radicle emergence seedlings were selected on the basis of the radicle length.

For the transmission electron microscopy (TEM) specimens were fixed in glutaraldehyde 3% in cacodylate buffer at pH 6.9 for 12 h, post-fixed in O_sO_4 1% for

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1 h, dehydrated in ethanol and embedded in Araldite. After staining with uranyl acetate and lead citrate, ultrahin sections were examined with a Hitachi 300 electron microscope at 75 kV. In order to enhance the ultrastructural details of the cell wall, the Periodic Acid–Thiocarbohydrazide–Silver Proteinate (PATAg) test (THIÉRY 1967) was performed on endosperm samples. Thin sections (1 µm) were stained with toluidine blue.

For scanning electron microscopy (SEM) samples, fixed and post-fixed as above, were dehydrated in an acetone series, critical point dried and coated with a gold film. Observations were made with a Cambridge Stereoscan 250 at 20 kV.

Chlorophylls were determined spectrophotometrically according to MORAN & PORATH 1980 using the extinction coefficients calculated by INSKEEP & BLOOM 1985.

Oxygen release was measured on small pieces of cotyledons with an oxygen electrode according to the method adopted by ISHII & al. 1977.

Results

The Seed.

When compared with *Picea abies* (DE CARLI & al. 1987), *Larix decidua* seeds had thicker coats and less abundant endosperm (Fig. 1). The seed coats were formed by several cellular layers, the outer of them having

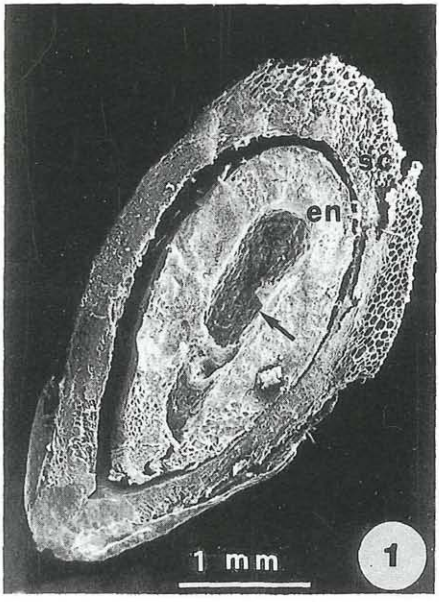




Fig. 1. SEM micrograph of a dissected dry seed. Seed coat (sc), endosperm (en) and the corrosion cavity (arrow) are visible.

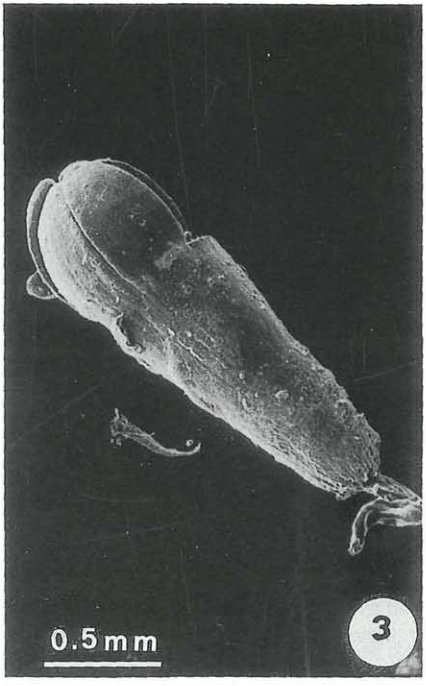
Fig. 2. Cross section of the seed coat. The cells with very thick walls are tightly packed; the first outer layer has small cells with thick outer tangential walls and electron-dense vacuolar content.

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smaller cells than the inner ones (Fig. 2). The walls were very thick, with a very compact fibrillar texture and the cells appeared empty. At the seed surface the cells had a very thick outer tangential wall and an electrondense vacuolar content, probably phenolic in nature. The thickness of the seed coat and of the cell walls accounted for the reduction of the transmitted light (40%), as evidentiated by measurements made with removed seed coats.

The embryo lay in the central "corrosion cavity", in close proximity to the gametophyte tissues; the large embryo axis bore 4–7 short and stumpy cotyledons (Fig. 3). Both embryo and endosperm stored fatty and proteinaceous reserves, respectively in lipid and protein bodies. Different amounts of proteins were stored in the cotyledons, axis and endosperm, the latter being clearly the most supplied with numerous and very large protein bodies. In the cotyledons more protein bodies were present in the cells surrounding the vascular bundle.

Storage cells of the sporophyte and gametrophyte tissues shared the same subcellular organization. Several electron-transparent lipid bodies filled the cytoplasm; the protein bodies had an electron-dense and



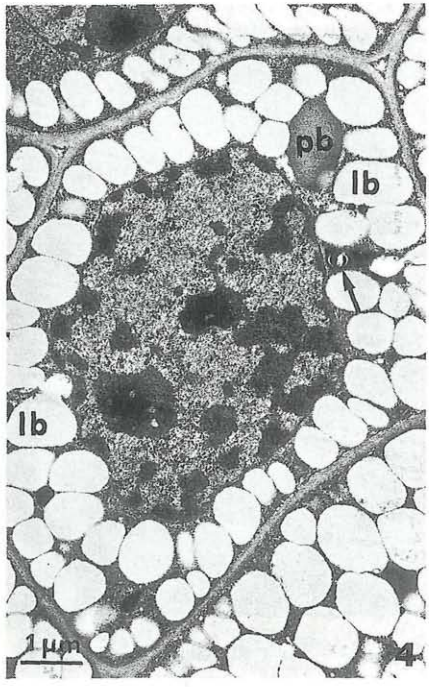


Fig. 3. SEM micrograph of an isolated embryo: the axis and the cotyledons are visible.

Fig. 4. Cotyledonal cell in an imbibed seed. The cytoplasm is filled with lipid bodies (1b); a protein body (pb) and a plastid (arrow) with a starch granule are also visible.

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homogeneous stroma in which an empty region, probably the globoid, could frequently be seen. Plastids with a small starch granule were infrequent. The ultrastructure of a storage cell is shown in Fig. 4, related to a cotyledon cell in an imbibed seed.

Seed Germination

Stratified and unstratified seeds show a different pattern of germination. The percentage of viable seeds which germinated within 10 days from sowing reached almost 100% in the stratified group while in the same time lag the same percentage fell to 77% in the other group, the remainder showing a delayed germination. Radicle emergence occurred 3 days after sowing in the stratified set, and after 4 days in the unstratified one. In 10 day old seedlings the radicle mean length was 9.85 ± 5.25 mm and 6.95 ± 4.99 mm, respectively. The relative variability (or coefficient of variation) was more than one third higher in the unstratified seeds vs. the stratified ones, thus indicating a less synchronous onset of germination.

A thin hypocotyl developed at first and then the cotyledons increased in length; they were covered by the seed coats and endosperm until about 15 days, but the zone inside the seed was gradually reduce during the growth, the apical region persisting to be covered last.

Reserve Breakdown

During the early stages of germination the stored reserves underwent a rapid hydrolysis. The process of degradation occurred at different rate in the sporophyte and gametophyte tissues. In the embryo the reserves were rapidly depleted in the axis cells, the hydrolysis starting already during the imbibing (Fig. 5). On the 4th day after sowing protein bodies were already digested, lipolysis was still in progress and starch was accumulated in the plastids (Fig. 6).

In the cotyledons the onset of the reserve hydrolysis was delayed, the protein digestion becoming evident only 3 days after sowing. The reserve degradation occurred faster in the cells surrounding the central vascular bundle (Fig. 8) with respect to the subepidermal cells (Fig. 7), as attested by the large vacuoles and the starch granules present in the former. When the cotyledon reserves were exhausted, the plastids of the inner cellular layer contained few thylakoids, and large starch granules were still present. Before the exhaustion of the gametophyte reserves, plastids filled with starch were also present in the epidermis and parenchyma close to the endosperm.

The endosperm cells underwent a gradual reserve breakdown, the first evidence of protein digestion being found 1 day after sowing. The hydrolytic activity did not occur at the same rate in all the cells, being from the beginning faster in the cellular layers lining the embryo. Consequently,

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these cells quickly exhausted the reserves and underwent a rapid degeneration, while in the outer cells under the seed coats the digestion was still in progress (Fig. 9). Only seldom were starch granules observed in the plastids.

An extensive lysis of the cell wall occurred simultaneously with the reserve breakdown, the matrix polysaccharides being preferentially digested. In the collapsed cells lining the corrosion cavity, where the cell walls were packed together, a loose microfibrillar network with empty meshes became evident (Fig. 10).

The depletion of all the reserves was completed within 15 days, and then the seed remainder was shed. In the stratified seeds the endosperm reserve mobilization occurred at a faster rate than in the unstratified ones.

Cotyledon Photosynthetic Activity.

During growth the cotyledons differentiated a functional photosynthetic machinery, as attested by the oxygen release, the chlorophyll content and the plastid ultrastructure. Table 1 reports the oxygen emission values and the respective chlorophyll content of different aged cotyledons. It is interesting to note that some photosynthetic activity was already present when

Table 1

Oxygen release and chlorophyll content in cotyledons of different ages.

Age days	Cotyledon region	O_2 release ($\mu mol \cdot h^{-1} \cdot g^{-1}$)	Chlorophyll (a + b) content ($mg \cdot g^{-1}$ FW)
5	–	–	0.490 ± 0.07
8	–	25.80 ± 3.40	1.090 ± 0.12
12	apical	49.40 ± 6.12	1.480 ± 0.14
	basal	92.10 ± 8.23	1.700 ± 0.15
16	apical	75.20 ± 8.30	1.780 ± 0.16
	basal	120.30 ± 10.45	2.040 ± 0.18

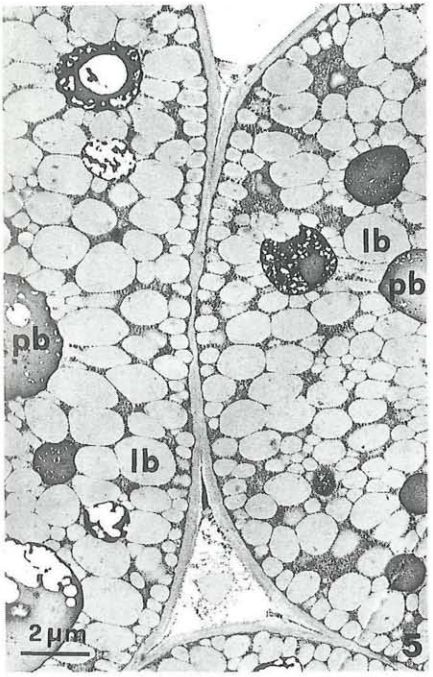
The values presented in the table represent the average of 5 measurements each made with 15 cotyledons.

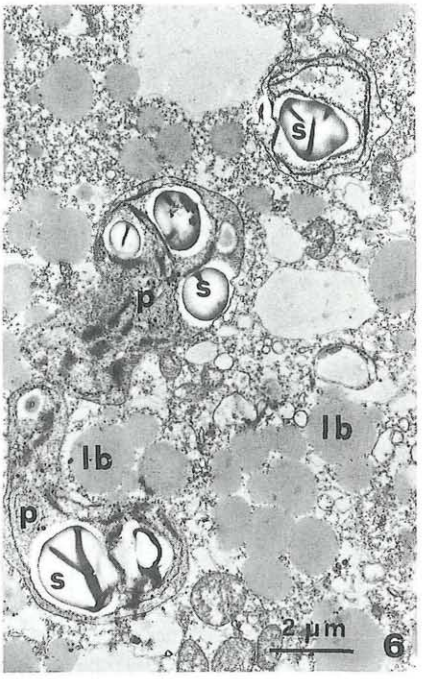
Fig. 5. Cells in embryonic axis of an imbibed seed: the protein digestion is started. (1b=lipid body; pb=protein body).

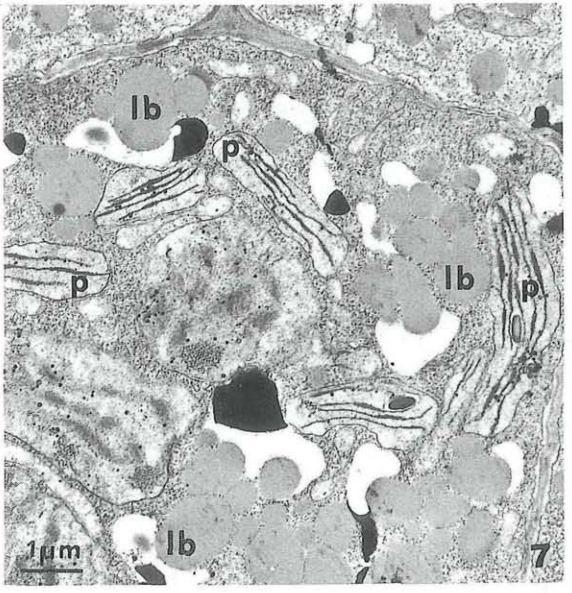
Fig. 6. Cell in embryonic axis 4 days after sowing. The protein bodies are digested; the lipolysis is in progress. Starch (s) is accumulated in the plastids (p). (1b=lipid body).

Figs. 7, 8. Cross sections of a cotyledon 6 days after sowing. The subepidermal cells (Fig. 7) have protein and lipid bodies partially digested and young plastids (p) with scarce and smalls tarch granules (s). In the innermost cellular layer (Fig. 8) large vacuoles (v) result from the advanced proteolysis.

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Fig. 9. Endosperm cell 6 days after sowing with many polisomes and rough RE. Mitochondria (m), glioxysomes (g) and plastids (p) are closely associated.

Fig. 10. Wall lysis in degenerated endosperm cells close to the embryo. The cells are collapsed and the walls closely packed. A loose fibrillar texture is evident in some cell wall. Remnants of the matrix are indicated by arrows. (PATAg test).

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young cotyledons (8 days old) were entirely enclosed in the seed tissues and the endosperm reserves were still abundant. Both oxygen release and chlorophyll content increased with age, with an evident functional difference between the apical and basal region of the cotyledons. The increase in functionality was paralleled by a gradual increase in the chloroplast membrane and stacking, as shown in Figs. 11–12. The tip-base gradient was recognizable also at the ultrastructural level, the apical cells being less differentiated than the basal ones.

Discussion

In *Larix decidua* cytological features during reserves breakdown are similar in both sporophyte and gametophyte tissues. They follow the pattern

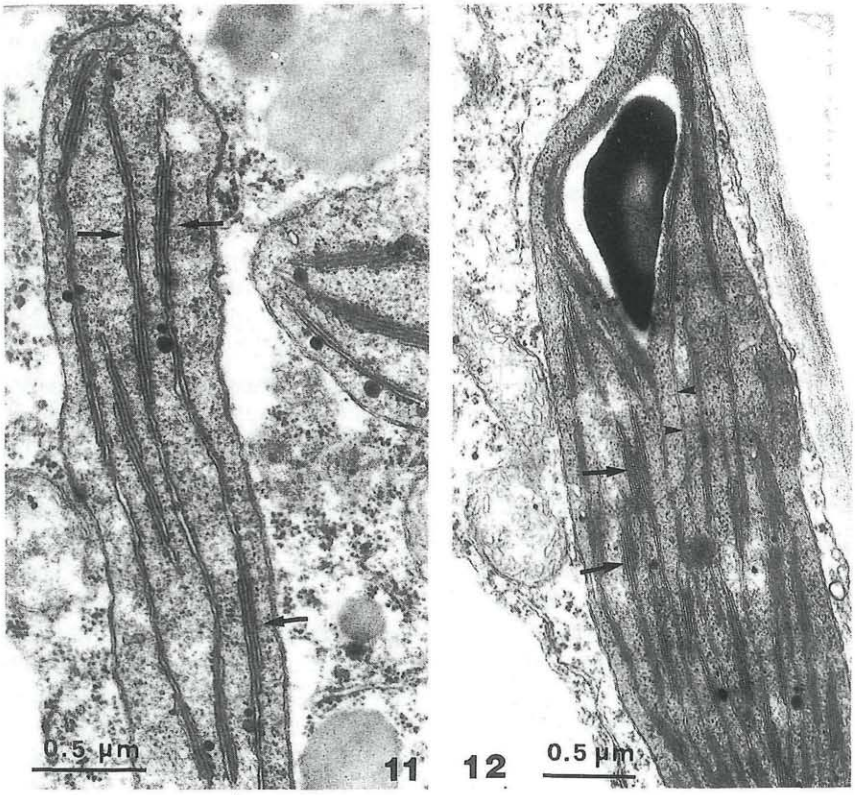


Fig. 11. Cotyledon 5 days from sowing: the young plastid has scarce thylakoids with irregular appressed regions (arrows).

Fig. 12. Mature chloroplast in 16 days old cotyledon with well developed thylakoid system arranged in granal (arrows) and stromatic (arrowheads) regions.

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already evidentiated in other Conifers (DURZAN & al. 1971, SIMOLA 1974 a, b, 1976, DE CARLI & al. 1987) and in *Ephedra distachya* (MARIANI & al. 1983).

The endosperm is the major storage organ, its reserves being broken down in an orderly way and the products being loaded by the cotyledons. We evidentiated an extensive degradation of the cell walls during reserve mobilization. It is likely that the cell walls act as a source of carbohydrates used for the embryo growth. At the same time cell wall degradation might make easier the apoplastic translocation of the digestion products from the endosperm to the cotyledons. Otherwise, as the reserves are gradually exhausted in the cells close to the embryo, the packed walls of the empty cells could finally form a thick barrier, thus slowing down the apoplastic efflux.

Usually embryo reserves attract scarce attention. The *L. decidua* embryo is equipped with abundant reserves. During germination, mainly in region with rapid growth, they are the early source of nutrients, are quickly metabolized and used *in situ*. SASAKI & KOZLOWKI 1969 state that during germination of *Pinus resinosa* seed the initiation of embryo growth does not depend on a supply of food from the gametophyte and that the utilization of the reserves in this tissue begin after the resumption of embryo growth. Our results suggest that the rapid axis growth is initially independent from the endosperm's nutrients: nevertheless the mobilization of reserves stored in the gametophyte is activated very early during germination. SALMIA & MIKOLA 1975, 1976a found relatively high activity of some peptidases in endosperm of *P. sylvestris* resting seeds.

The cotyledons are highly specialized leaves whose functional competence changes during germination. As in other Conifers they last several months and support the seedling growth by using at first their own reserves, and by actively traslocating the hydrolytic products coming from the endosperm. Furthermore early during germination they gain photosynthetic functionality, so that they become able to satisfy the nutritional requirement of the whole seedling when the maternal reserves are exhausted. During germination the cotyledonal plastids show a certain local specialization. Initially they actively participate in the reserve metabolism. Plastids close to the endosperm and to the vascular bundle are rich in large starch granules. This feature is maintained in plastids of the innermost cells also in photosynthesizing cotyledons, and couples with a scarce equipment of thylakoids. This finding is in accordance with the CASADORO & RASCIO 1987 report on Clementine green cotyledons. Early during differentiation plastids on the side opposite to the endosperm acquire a well developed set of thylakoids, with granal and intergranal organization and probably account for the initial photosynthetic activity. Later, when the endosperm reserves are exhausted and the seed remainder is shed, all the outer mesophyll plastids are photosynthetically active.

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As previously presumed for *P. abies* (DE CARLI & al. 1987), the persistence of the endosperm over the apical region of the cotyledons could affect the rate of cellular differentiation through a direct control as well as through microenvironmental conditions.

The ordered sequence of events involved in mobilization of stored reserves in gametophyte (endosperm) and sporophyte (axis and cotyledons) points to an endogenous control. An experimental approach we made with excised endosperms (data not shown) seems to corroborate a control of the embryo on the reserve breakdown. Several papers concern the control by the embryo on the reserve breakdown in dicotyledonous seeds (see, for example, DAVIES & CHAPMAN 1979a, b, GIFFORD & al. 1984, CHAPMAN & GALLESCHI 1985, REVILLA & FERNÁNDEZ-TÁRRAGO 1986, TARPLEY & CHOINSKY 1986); indications exist also for Conifer seeds (NYMAN 1971, BILDERBACK 1974, MURRAY & ADAMS 1984). The two alternative hypotheses to explain how the control is exerted (viz. hormonal control and source-sink effect) have been reviewed by DAVIES & SLACK 1981 and, more recently, by CHAPMAN & DAVIES 1983: these latter envisage as the most acceptable hypothesis the second one. A short period of cold stratification allows the *L. decidua* seeds to overcome the mild dormancy (RUDOLF 1979), enhancing both the rate of germination and the radicle elongation, as well as the rate of endosperm reserve mobilization. According to the results by CARPITA & al. 1983 and MURPHY & HAMMER 1988 with *Pinus* species, stratification induces a higher rate of reserve mobilization in the endosperm through an increased requirement of nutrients by the embryo (increase of growth potential of the sink) rather than through a direct effect of cold treatment on the gametophyte itself.

During reserve mobilization and utilization a rise in activity of the key enzymes must be expected. Some information comes from papers of SALMIA & MIKOLA 1975, 1976 a, b, NOLAN & MURPHY 1984, who considered biochemical aspects of the germination in some *Pinus* species. Recently PITEL & CHELIAK 1986 examined the changes in enzyme activity of both endosperm and embryo during imbibing and germination of *L. laricina* seeds. The timing of enzyme activity seems to coincide well with our cytological observations on the rate of reserve breakdown and on the seedling growth. A suggestion comes from our ultrastructural data: for a complete understanding of the biochemical events during germination in Conifer species it could be useful to include the wall degrading enzymes in the screening of the enzymatic activity of the endosperm.

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