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Spatio-temporal variations in starch accumulation during germination and post-germinative growth of zygotic and somatic embryos of *Pinus pinaster*

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Abstract

During germination and post-germinative growth of *Pinus pinaster* Ait. seeds, triglycerides are hydrolysed and concurrently the embryo accumulates starch. In this study, the spatio-temporal variation of starch accumulation was described in zygotic embryos associated (ZE^+) or not (ZE^-) to their megagametophyte and in somatic embryos (SE). In germinating ZE^+, starch was accumulated in the growing tissues, following closely the spatio-temporal pattern of triglycerides depletion. In contrast, in ZE^- and SE, starch was only found in cortical cells close to the culture medium. In germinating ZE^+, the spatio-temporal variations of starch accumulation can be thus interpreted as the result of the changing contact between the megagametophyte and the growing tissues and also of the existing interactions between triglyceride hydrolysis and the allocation of sucrose exported from the megagametophyte.

Additional key words: megagametophyte, pine, sugar distribution, triglycerides.

Introduction

In oil-storing seeds, conversion of lipids to saccharides is triggered by germination and begins with the hydrolysis of triglycerides to free fatty acids. These then enter the glyoxysomes where they provide substrates for the β-oxidation reactions and the glyoxylate cycle which finally contributes to the gluconeogenesis in the cytosol. The released glycerol can enter the glycolytic pathway or participate to the gluconeogenesis as well (Mohr and Schopfer 1995). In pine seeds, the mature embryo contains triglycerides and arginin-rich protein bodies spread throughout all tissues (Ching 1966, King and Gifford 1997, Stone and Gifford 1997). In contrast, only a few small starch grains can be detected. During germination and post-germinative growth, the triglycerides stored in the oleosomes are progressively depleted and new synthesised saccharides are accumulated in amyloplasts (Murphy and Hammer 1994, Stone and Gifford 1999). The spatio-temporal pattern of lipid depletion has been described (Jordy *et al.* 2000). It occurs progressively during elongation of the preformed tissues of the embryo, first in the growing radicle, then in the hypocotyl and cotyledons. The shoot apical meristem (SAM) is the last site to retain lipid reserves. Reserves contained in the megagametophyte are similar to that of the embryo. They are hydrolysed following the same pathway as described above, but no starch storage occurs (Stone and Gifford 1999).

In contrast to triglycerides, the spatio-temporal pattern of saccharide distribution has not been much investigated. Soluble and insoluble sugars have been quantified and presence of amyloplasts has been observed in the cortical parenchyma of germinating embryos of *Pinus taeda* (Stone and Gifford 1999), but their distribution in growing seedling remains unknown. Concerning somatic embryos, the more advanced data available concern the maturation of zygotic and somatic embryos of *Picea abies,* in which dynamics of non-structural saccharides, starch and key enzymes of sucrose metabolism have been described using high performance liquid chromatography combined with histochemical detection (Gösslová *et al.*

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Abbreviations: SAM - shoot apical meristem; SE - somatic embryo; ZE^+ - zygotic embryo associated with its megagametophyte; ZE^- - zygotic embryo deprived of its megagametophyte.

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2001, Konrádová *et al.* 2002). In the present paper, we report the results of an histological investigation of starch accumulation during the germination and the post-germinative growth of *Pinus pinaster* zygotic embryos associated (ZE^+) or not (ZE^-) with their megagametophyte and of somatic embryos (SE). The main objective was to gain a better understanding of sugar allocation and embryo-megagametophyte interactions during these early steps of plant development.

Materials and methods

Seeds of *Pinus pinaster* Ait. were cultured in the dark at 25 °C on moistened filter paper in Petri dishes. After radicle emergence, they were exposed to a 16-h photoperiod (irradiance of 350 µmol m^{-2} s^{-1}). Ten zygotic embryos or germinating seedlings associated with their megagametophyte (ZE^+) were collected before imbibition (= dehydrated embryos), at imbibition (4 d - stage 1), radicle emergence (7 d - stage 2) (Fig. 1), hypocotyl elongation (14 d -- stage 3) (Fig. 2), expansion of the cotyledons (21 d - stage 4) and of the first primary needles (28 d - stage 5). In parallel, 6 to 10 zygotic embryos deprived of their megagametophyte (ZE^-) and mature somatic embryos (SE) (AFOCEL clone T325) cultivated on a solidified culture medium (Margara 1977) containing 2 % (m/v) sucrose were harvested at stages 2 and 4.

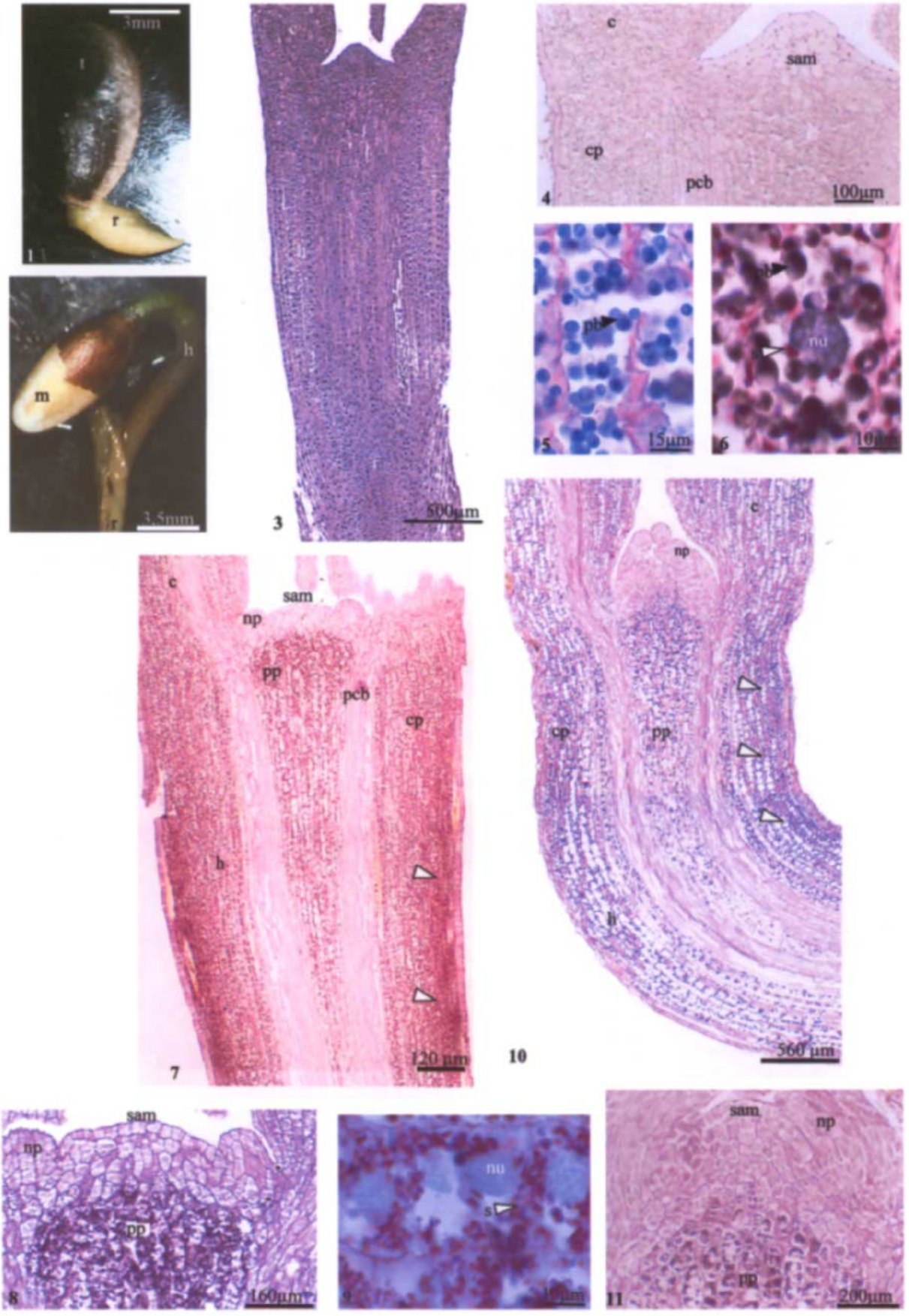
Seedlings and emblings were fixed in the *CRAF* solution (Randolph 1935) and embedded in *Paraplast* following traditional procedures. To reveal sugars, longitudinal sections (9 µm) were purplish pink stained with Schiff reagent after oxidation with periodic acid (PAS). Complementarily, in some sections protein bodies were visualised in deep blue using *Amido-Black 10B* staining (Clark 1981). Triglycerides have been also revealed in ZE^- and SE using *Oil red O* as described in Jordy *et al.* (2000). Observations were carried out on images captured using microscope (*Nikon Optiphot 2*, Tokyo, Japan) equipped with a digital camera (*Nikon D1*) coupled with *Visilog 5.1* (*Noesis*, Courtaboenf, France) software.

Results

Dehydrated ZE^+ contained protein bodies distributed throughout all tissues, but starch grains were not detected (Fig. 3, 4, 5). At stage 1, few small starch grains (1.9 ± 0.7 µm) (Fig. 6) were observed in all tissues of ZE^+. Then, diameter and abundance of starch grains increased: *a*) at stage 2, in the radicle and in the lower part of the hypocotyl (Figs. 7, 8, 9); at this stage, the megagametophyte was still covering the whole germinating embryo, except the emerging radicle (Fig. 1); *b*) at stage 3, in the whole hypocotyl, especially in its apical part (Figs. 10,11); at this stage, the hypocotyl had eluded contact with the megagametophyte which is progressively moved to the shoot-tip of seedlings (Fig. 2), and *c*) at stage 4, in the cotyledons which remained the last tissues in contact with the megagametophyte.

Starch was more abundant in the cortical parenchyma than in the pith (Figs. 7, 10). Within the shoot-apex, the pith parenchyma underlying the SAM stored high amount of amyloplasts from stage 2 to stage 4 (Figs. 8, 11). The SAM itself still remained poor in polysaccharide reserves. At stage 5, starch was no longer detected (Figs. 12, 13). At this stage, elongation of the preformed tissues of the embryo was completed, the megagametophyte discarded, and the first primary needles in expansion.

Figs. 1 - 11 (*following page*).
Fig. 1. Germinating seed at stage 2.
Fig. 2. Germinating seed at stage 3; shoot-apex and cotyledons covered by the megagametophyte.
Fig. 3. Dehydrated ZE^+ - abundant protein bodies in all tissues, but no starch grains detected.
Fig. 4. Dehydrated ZE^+ - detailed view of the shoot-apex.
Fig. 5. Dehydrated ZE^+ - protein bodies in pith parenchyma cells.
Fig. 6. Stage 1 - ZE^+ - protein bodies and newly synthesised starch grains in pith parenchyma cells.
Fig. 7. Stage 2 - ZE^+ - starch accumulation in the cortex of the hypocotyl especially at distance from the apex (*arrows*), and in the pith parenchyma below the shoot-apex.
Fig. 8. Stage 2 - ZE^+ - detailed view of the shoot-apex; starch abundant in the pith parenchyma underlying the SAM.
Fig. 9. Stage 2 - ZE^+ - cortical parenchyma cells with numerous starch grains stored.
Fig. 10. Stage 3 - ZE^+ - starch grains especially abundant in the apical part of hypocotyl (*arrows*).
Fig. 11. Stage 3 - ZE^+ - detailed view of the shoot-apex; starch grains abundant in the pith parenchyma underlying the SAM.
Abbreviations: r - radicle, m - megagametophyte, t - tegument, h - hypocotyl, c - cotyledon, e - epicotyl, sam - shoot apical meristem, cp - cortical parenchyma, pp - pith parenchyma, pcb - procambium, np - needle primordia, n - elongating primary needles, s - starch, pb - protein body, nu - nucleus. Staining: PAS - Figs. 4, 7, 8, 11; PAS + *Amido-Black 10B* - Figs 3, 5, 6, 9, 10. Sugars and proteins were purplish pink and deep blue stained, respectively.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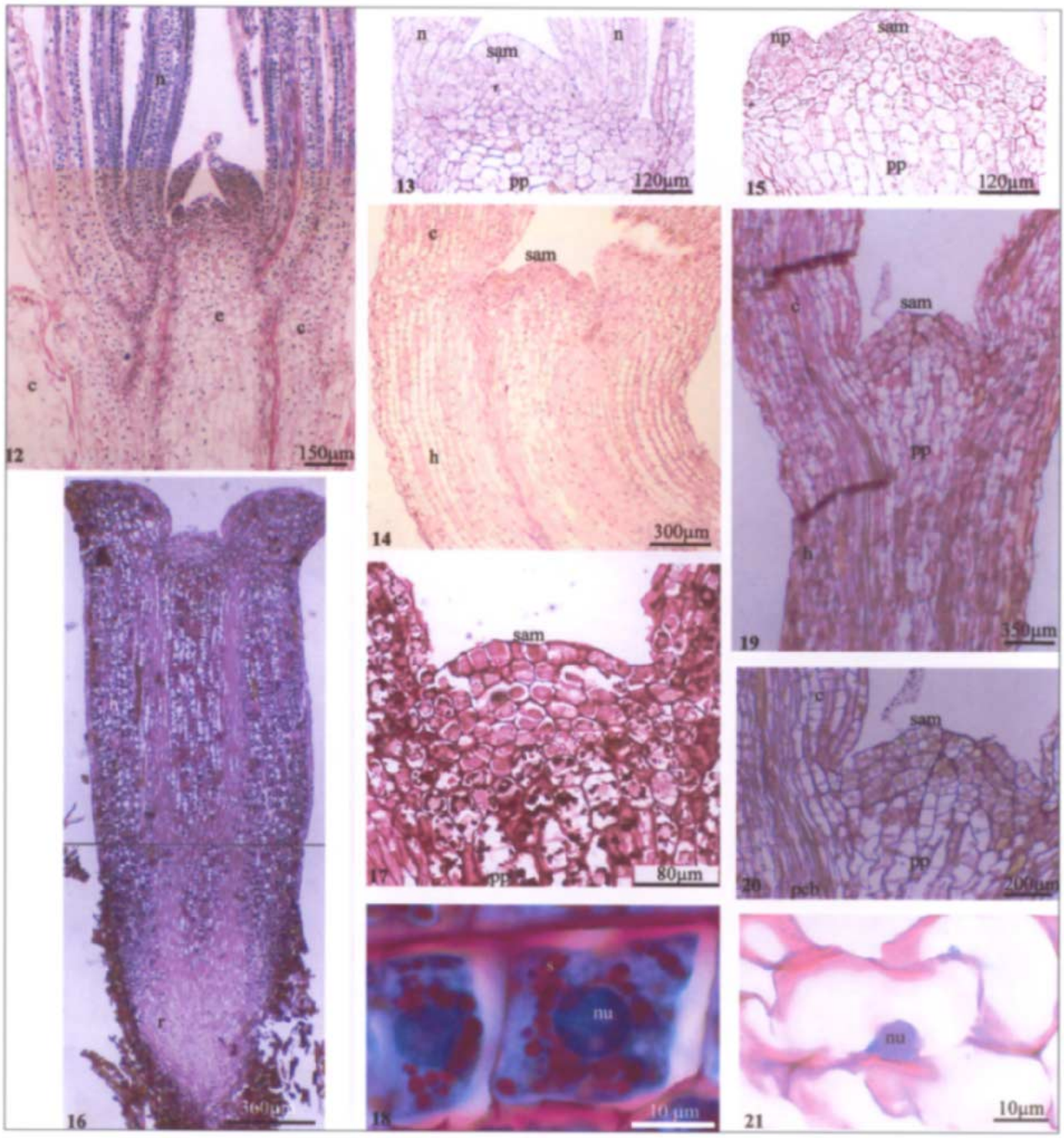

Fig. 12. Stage 5 - ZE^+ - no starch detected in epicotyl and elongating primary needles.
Fig. 13. Stage 5 - ZE^+ - detailed view of the shoot-apex; no starch grain detected.
Fig. 14. Stage 2 - ZE^- - no starch detected in hypocotyl and cotyledons.
Fig. 15. Stage 2 - ZE^- - detailed view of the shoot-apex; starch grains missing in the sub-apical pith parenchyma.
Fig. 16. Dehydrated SE - abundant starch grains in pith and cortical parenchyma.
Fig. 17. Dehydrated SE - detailed view of shoot-apex; starch accumulation in the sub-apical pith parenchyma and cotyledons.
Fig. 18. Dehydrated SE - starch grains accumulated in cortical parenchyma cells.
Fig. 19. Stage 2 - SE - no starch detected in hypocotyl and cotyledons.
Fig. 20. Stage 2 - SE - detailed view of the shoot-apex; no starch detected.
Fig. 21. Stage 2 - SE - parenchyma cells empty of sugar reserves.
Staining: PAS - Figs. 15, 16, 17, 18, 19, 21, 22; PAS + *Amido-Black 10B* - Figs. 12, 13, 14, 20, 23. For other detail see legend to Figs. 1 - 11.

In ZE^-, starch storage did not occur (Figs. 14, 15). A few amyloplasts could only be observed at stage 2, exclusively in the cortical parenchyma close to the culture medium. Triglycerides were depleted as observed in ZE^+ (not shown) (Jordy *et al.* 2000). At stage 4, the hypocotyl length was 1.5 cm instead of 4 cm in ZE^+.

Contrasting with the zygotic embryos, the dehydrated mature SE contained plenty of starch reserves (Figs. 16, 17, 18). Triglycerides were present but less abundant than in ZE (not shown). During radicle and hypocotyl elongation, starch completely disappeared except in few parenchyma cells located in close contact with the culture medium (Figs. 19, 20, 21). Triglycerides were depleted following the same pattern as in ZE^+. At stage 4, the hypocotyl length was about 1.5 cm, as in ZE^-.

Discussion

Among the 3 types of studied embryos, only SE exhibited abundant starch reserves before germination. This observation can be related to the transitory accumulation of starch occurring before triglycerides formation during maturation of rape seeds (Da Silva *et al.* 1997). The point has to be checked in the case of pine seeds, but the low amount of triglycerides detected in mature SE of *Pinus pinaster* is compatible with this hypothesis.

During germination and post-germinative growth, only ZE^+ abundantly synthesised starch. This starch accumulation surprisingly occurred during expansion of the preformed tissues of the embryos which became strong sinks during their development: it began in the emerging radicle, continued in the growing hypocotyl, and finished in the expanding cotyledons.

This spatio-temporal pattern of starch accumulation seems to be influenced by the embryo-megagametophyte interactions. Indeed, after radicle emergence the megagametophyte is progressively shifted to the cotyledons tip. During this movement, the growing radicle, hypocotyl and cotyledons successively loose contact with this reserve tissue, which represents an essential source of sucrose from germination to a late stage of post-germinative growth (Stone and Gifford 1999).

Within ZE^+, starch accumulation was particularly abundant in the cortical parenchyma and in the pith meristem underlying the shoot apex. The reserves stored in this sub-apical zone most likely contribute to sustain the organogenetic activity of the SAM which is particularly intense at stages 2 to 4. In the seedling axis, the cortical parenchyma is together with the epidermis, in close contact with the megagametophyte and directly involved in accumulation of imported sugars.

In ZE^- and SE, the lack of starch accumulation in the cortical parenchyma except nearby the sucrose-enriched medium suggests that translocated sugars are accumulated in the nearest parenchyma cells just after their absorption. Furthermore, it has been evidenced that sugar supply under *in vitro* conditions may have effects on future plant development (Lipavská and Voráčková 1994). Thus, the reduced sized hypocotyl at stage 5 likely indicated that increasing surface of contact between embryos and culture medium may have positive effects on starch storage in the embryo and thus to its development.

Furthermore, it is known that soluble sugar synthesis in germinating oil-seeds is connected to the breakdown of lipid reserves that provides substrates for the gluconeogenesis (Mohr and Schopfer 1995). Interestingly, the spatio-temporal pattern of starch accumulation revealed by the present results fits in with that previously described for lipid depletion (Jordy *et al.* 2000). Both phenomena occur on the same sites, and at the same time, thus suggesting that starch accumulation in growing pine embryos is linked to triglycerides breakdown.

Several authors however demonstrated that substrates for starch synthesis are provided by the megagametophyte. Murphy and Hammer (1994), for example, showed that, in *Pinus taeda,* introduction of ^{14}C sucrose in the megagametophyte resulted in starch labelling in the embryo. It has been shown in addition, that during germination the glyoxylate cycle is inhibited in the embryo but not in the megagametophyte, leading to fatty acid production and energy releasing for the growing tissues (Mullen and Gifford 1995a, 1995b, Stone and Gifford 1999). Starch synthesis in the embryo is thus mainly issued from sucrose translocated from the megagametophyte and not from local conversion of triglycerides to sugars. These sugars are transitory stored in amyloplasts on the site of triglycerides hydrolysis before being oxidised. Such paradoxical storage in sink tissues results probably from the quick weaning of the seedling from its megagametophyte.

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