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THE ACTIVITY OF SOME ENZYMES OF LIPID METABOLISM IN SILVER FIR SEEDS (*ABIES ALBA* MILL.) DURING GERMINATION

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The activity of some enzymes of lipid metabolism in silver fir seeds (*Abies alba* Mill.) varied in different ways in the embryo and the endosperm during germination. The activity was followed in the seeds from the Mašun region, which did not germinate at room temperature, and in the well germinated seeds from the Papuk region.

Lipase activity was measured at pH 8.5 (alkaline lipase) and at pH 5.5 (acid lipase). Mature seeds of both proveniences showed acid and alkaline lipase activity. As the activity of alkaline lipase in the endosperm did not change in any appreciable way before and during visible germination, we concluded that the alkaline lipase activity is not a limiting factor in germination. The activity of acid lipase and catalase increased somewhat after the penetration of the radicle in the well germinated seeds, while the activity of isocitrate lyase increased greatly. On the other hand, the activity of all enzymes assayed showed a constant decrease in the endosperm of the seeds from the Mašun region.

Acid and alkaline lipase and catalase were active before visible germination. Their activities were almost undetectable after radicle emergence while the activity of the nongerminated seeds from the Mašun region stayed constant (acid lipase, catalase) or showed a slow decrease (alkaline lipase). The embryo showed no isocitrate lyase activity.

The presence of microbodies was also examined in the electron microscope. Glyoxysomes were found alredy in the endosperm of mature seeds. Their number increased with germination. Microbodies were also present in the embryo before and during visible germination. It is suggested that the organelles under consideration do not function like glyoxysomes as there was no isocitrate lyase present in embryos. We conclude that in the embryo the lipids are not metabolised via the glyoxylate cycle as is true for the endosperm.

Introduction

In oil seeds a substantial portion of dry weight comprises storage triacylglycerols. The primary stage in fat utilization is commonly supposed to be its hydrolytic fission to glycerol and free fatty acids under the action of lipase. Liberated fatty acids are then converted to sugars via β-oxidation and the glyoxylate cycle (Bewley and Black 1978). The evidence now available shows that activation of fatty acids, their oxidation to acetyl CoA, and the conversion of acetyl CoA to succinate occurs in glyoxysomes, that the conversion of succinate to malate occurs in the mitochondria and the conversion of malate to sucrose in the cytosole (Cooper and Beevers 1969, Hutton and Stumpf 1969, Nishimura and Beevers 1979). In 1967 Breidenbach and Beevers precisely determined the location of the two key glyoxylate cycle enzymes, isocitrate lyase and malate synthetase in glyoxysomes. The activities of the glyoxylate cycle enzymes increase steadily after the onset of germination, than decline rapidly as triacylglycerols reserves are depleted (Gerhardt and Beevers 1970, Schnarrenberger et al. 1971).

In silver fir seeds the haploid gametophyte enveloping the embryo is the storage tissue where reserve substances are hydrolized, converted, and translocated to the embryo for synthesis of various cellular constituents. Lipids are the major food reserves in both the gametophyte and the embryo. Lipids are found to comprise 25% of fresh weight and 44% of dry weight, the endosperm containing 90% and the embryo 10% of lipids. The decomposition of reserve triglycerides begins in already germinating seeds and is associated with an increase in the amounts of soluble sugars (Kovač and Vardjan 1981).

The present investigation was designed to determine the activity of some enzymes of lipid metabolism prior to and during visible germination and to compare this enzyme activity from well germinated silver fir seeds to nongerminated ones.

Materials and Methods

Seed germination

Silver fir seeds (*Abies alba* Mill.) collected in 1980 in the Mašun region (NE Snežnik Mt., Notranjsko, Slovenija), 1020 m above sealevel and in 1980 in the Papuk region (Papuk Mt., Croatia), 500—660 m above sea-level, were used in the experiments. After 24 hrs imbibition in distilled water at room temperature (25° C) the seeds were germinated on wet filter paper in Petri dishes under white fluorescent light (Sylvania lights at 32 W m^{-2}, 12 hrs night, 12 hrs day) at 22—28° C. Another set of seeds was imbibed in water for 24 hrs at + 3° C and subsequently stored for 21 days in constantly moist vermiculite at the same temperature. After stratification the seeds were transferred to room temperature (stratified seeds). Seeds from the Snežnik region were also set for germination in 2l Erlenmayer flasks, filled with 1% aqueous solution of H_2O_2 (200 seeds in 600 ml of 1% H_2O_2). Hydrogen peroxide was suggested for the rapid viability test of some coniferous tree seeds (Ching and Parker 1958, Simak 1968).

Preparation of enzyme extract

At selected time-intervals the seeds were carefully decoated and embryos were separated from endosperms. Fifty endosperms and 150 embryos were ground vigorously with a prechilled mortar and pestle in 10 ml of a grinding medium containing 0.1 mol l^{-1} Tris-HCl pH 7.5, 1 mmol l^{-1} EDTA, 10 mmol l^{-1} 2-mercaptoethanol and 1 mmol l^{-1} $MgCl_2$. The extracts were further homogenized with a motorized Teflon pestle. Samples to be tested for catalase activity were homogenized in the same manner in only 0.1 mol l^{-1} Tris-HCl buffer at pH 7.5. The homogenates were centrifuged at 10.000 g for 20 min before the analyses were made. The resultant supernatants were filtered through a filter for coarse precipitates to remove the floating fat layer. The clarified filtrates were used for enzymatic assays. All the procedures were carried out at 4° C. The samples were stored at —25° C.

Enzyme assays

Lipase activity was measured by the amount of p-nitrophenol released from p-nitrophenyl caprate (Sigma Chemical Co.) (Marriott and Northcote 1975). We used 0.1 mol l^{-1} citric acid — Na_2HPO_4 buffer, pH 5.5 instead of 40 mmol l^{-1} sodium acetate buffer, pH 5. The absorbance at 410 nm was measured against a reference sample which was made in the same manner as the sample except that trichloroacetic acid was added before the enzyme extract.

Isocitrate lyase activity was determined by the modified spectrophotometric assay of Dixon and Kornberg (1959). The reaction mixture contained in a final volume of 3 ml 0.1 mol l^{-1} Tris-HCl buffer, pH 7.1 : 15 μmol $MgCl_2$, 10 μmol of phenylhydrazine hydrochloride (prepared fresh each day), 22 μmol of 2-mercaptoethanol and 0.2 ml of enzyme extract. The reaction was started by the addition of 10 μmol of DL-isocitric acid (Fluka AG) and monitored by continuous recording at 324 nm and 30° C against a reference sample in which the substrate was omitted.

Catalase activity was assayed by the initial disappearance of 10 mmol l^{-1} H_2O_2 as measured by the decrease in absorbance at 240 nm and 30° C (Aebi 1974). The complete reaction mixture contained in a volume of 3 ml, 50 mmol phosphate buffer, adjusted to pH 7.5, freshly prepared 10 mmol hydrogen peroxide (Zorka Šabac) and 0.1 ml of the enzyme extract.

All the enzyme assays were carried out using a Beckman, Acta IIC recording spectrophotometer. For the endosperm extract the data shown were the mean values of three experiments and three determinations. For the embryo extract there was only one representative experiment and each value was the average of three determinations.

Electron microscopy

Segments of endosperms and embryos obtained at each germination stage were fixed for electron microscopy as described by Vigil (1970) and after dehydration embedded in Araldite. Thin sections were stained with uranyl acetate and lead citrate. The method of incubating tissue in DAB (3,3'-diaminobenzidine, Sigma Chemical Co.) described by Frederick and Newcomb (1969) was used to detect catalase cytochemically. The controls were preincubated in aminotriazole (3-amino-1,2,4-triazole) and then incubated in the standard DAB medium.

Light microscopy

For light microscopy, 1 μm sections of Araldite embedded tissue were stained with 2% toluidine blue before examination with phase optics.

Results

Seed germination

Silver fir seeds (*Abies alba* Mill.) from the Mašun region did not germinate at room temperature, while the seeds from the Papuk region did germinate. Three week stratification had no influence on the percentage of germination of seeds from Sneznik as shown in Fig. 1, while the same seeds gave the same germination value as stratified seeds from the Papuk region, when germinated in a 1% aqueous solution of hydrogen peroxide. We concluded that the seeds from Mašun were viable but in deep dormancy.

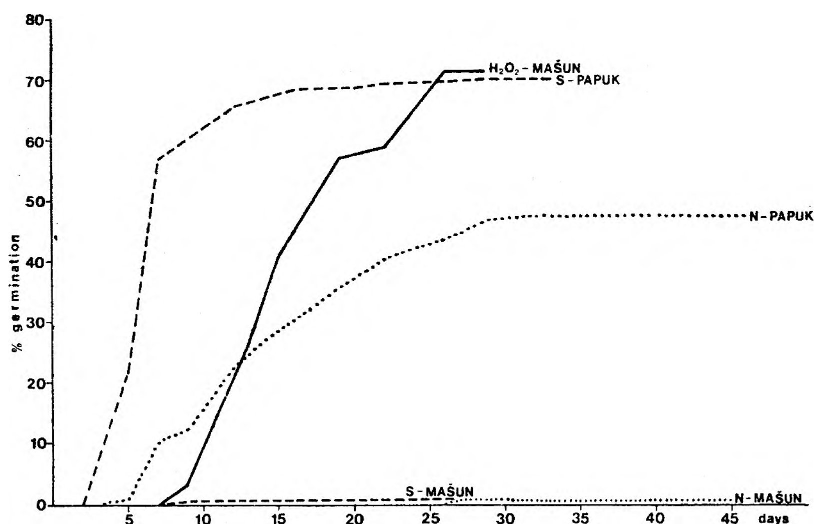


Fig. 1. Germination curves of silver fir seeds from the Papuk region and the Mašun region. N = nonstratified seeds, S = 21 days stratified seeds, H_2O_2 = seeds germinated in a 1% aqueous H_2O_2.

Development of enzyme activity

Lipase. The clear endosperm and embryo extract from mature silver fir seeds showed both acid and alkaline lipase activity (pH 5.5 and pH 8.5) (Fig. 2). The lipase activity was absent in the floating lipid-body layer. The enzyme activity was higher in the endosperm than in the embryo. The highest activity was shown by the alkaline lipase in the endosperm but its value did not change considerably during germination.

The acid lipase showed only a slight increase in activity at the time of radicle emergence. In the embryo too, the alkaline lipase was more active than the acid one. It decreased to a negligible amount in the embryo with a 2—3 cm long radicle. This was also the case with the acid lipase. These results may suggest that the activity of lipases in the embryo are important for the induction of germination and not for the rapid growth of the young embryo.

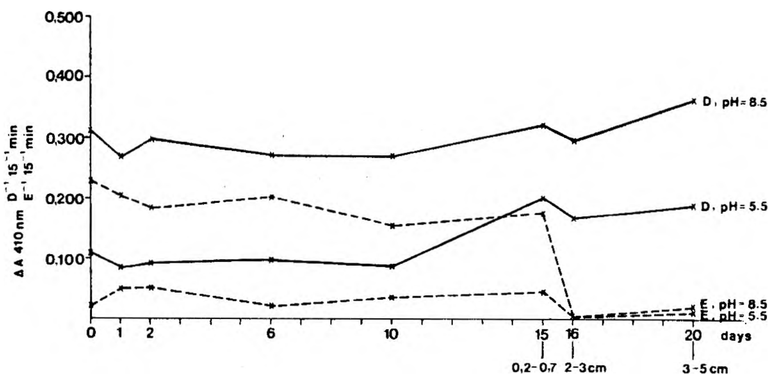


Fig. 2. Acid and alkaline lipase activity in silver fir seeds from the Papuk region. E = embryo, D = endosperm. The numbers on the 15th, 16th and 20th day indicate the radicales length of the germinating seeds.

We also measured the activity of acid and alkaline lipase in the seeds from the Mašun region, which did not germinate throughout the experimental period (Fig. 3). Their activity was even higher in the endosperm of mature seeds than in the seeds from the Papuk. This was not true for the alkaline lipase in the embryo which proved to be important for the induction of germination. Lipase activity decreased greatly in the seeds which remained at room temperature for 20 days. These seeds already showed signs of decay.

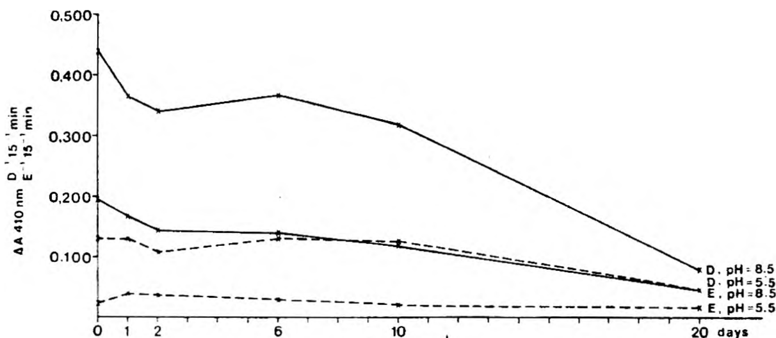


Fig. 3. Acid and alkaline lipase activity in silver fir seeds from the Mašun region. E = embryo, D = endosperm.

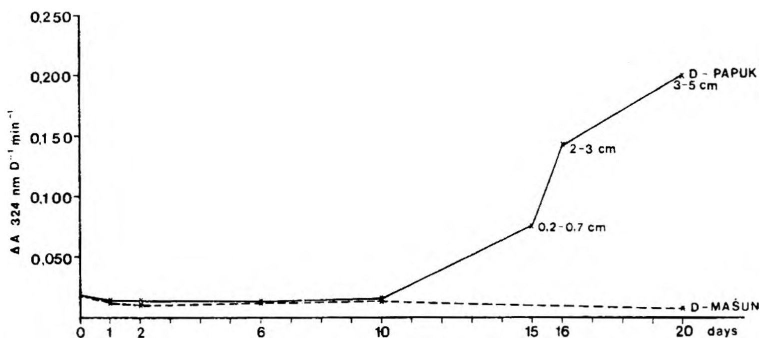


Fig. 4. Isocitrate lyase activity in silver fir seeds from the Papuk and the Mašun region. D = endosperm. The numbers on the 15th, 16th and 20th day indicate the radicles length of the germinating seeds.

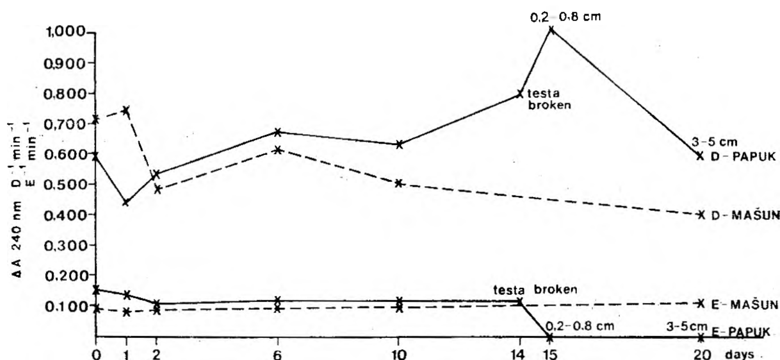


Fig. 5. Catalase activity in silver fir seeds from the Papuk and the Mašun region. E = embryo, D = endosperm. The numbers on the 15th and 20th day indicate the radicles length of the germinating seeds.

Figs. 6—13. Endosperm and embryo cells after incubation in DAB medium. mb = microbody, lb = lipid body, pb = protein body, s = starch, m = = mitochondrion, d = dictyosome, n = nucleus, v = vacuole, p = plastid.

Fig. 6. Seed endosperm after 24 hr incubation. 17,000 : 1.

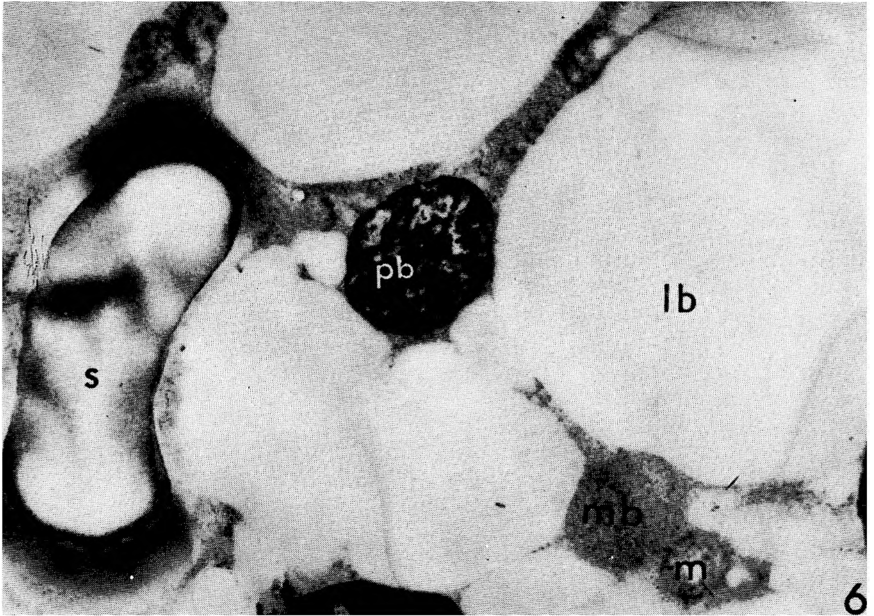
Fig. 7. Seed endosperm with a 0.2—0.4 cm long radicle. 17,000 : 1.

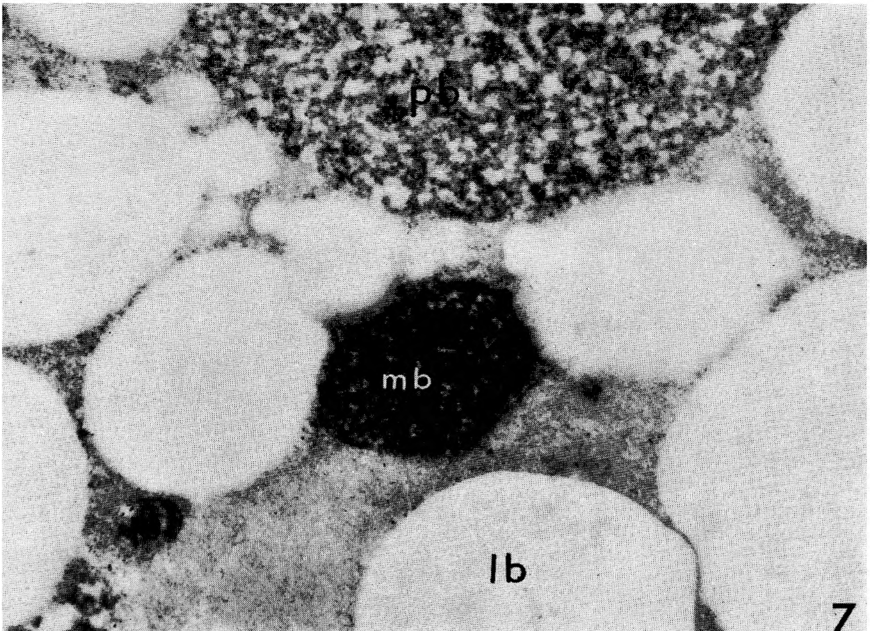
Figs. 8 and 9. Seed endosperm with a 1.5—2 cm long radicle. 17,000 : 1.

Fig. 10. Seed embryo after 24 hr incubation. 24,000 : 1.

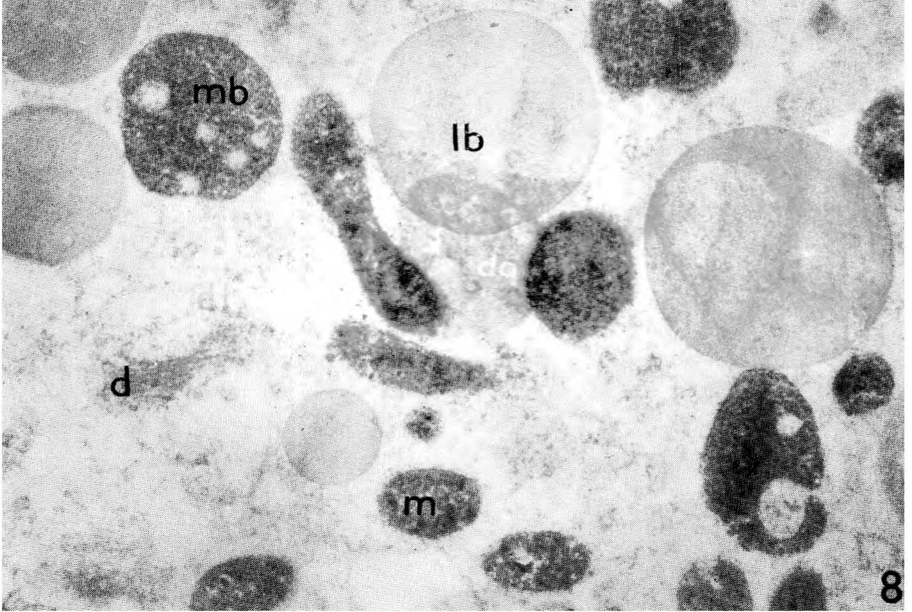
Fig. 11. Seed embryo with a 0.2—0.4 cm long radicle. 17,000 : 1.

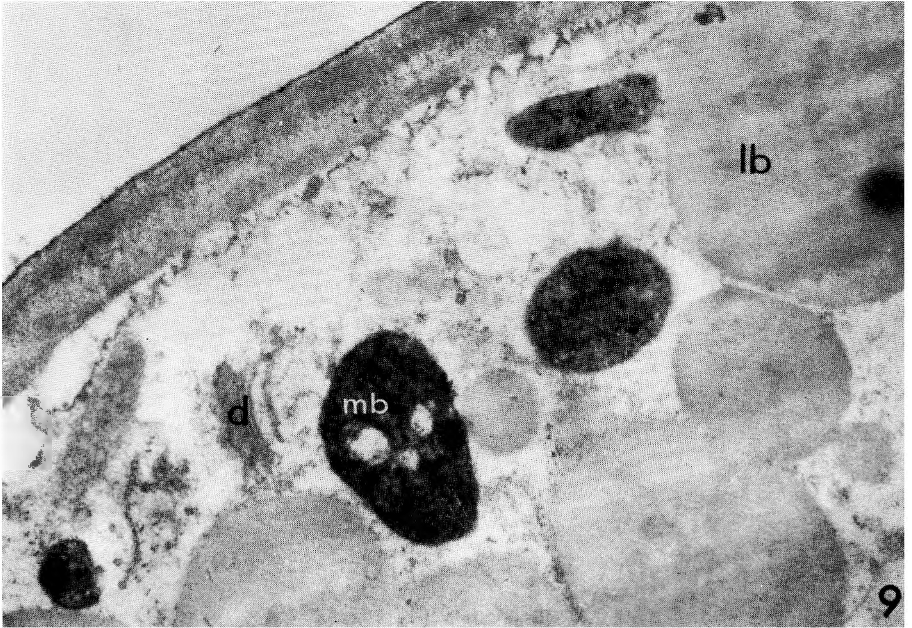
Figs. 12 and 13. Seed embryo with a 1.5—2 cm long radicle. 17,000 : 1.



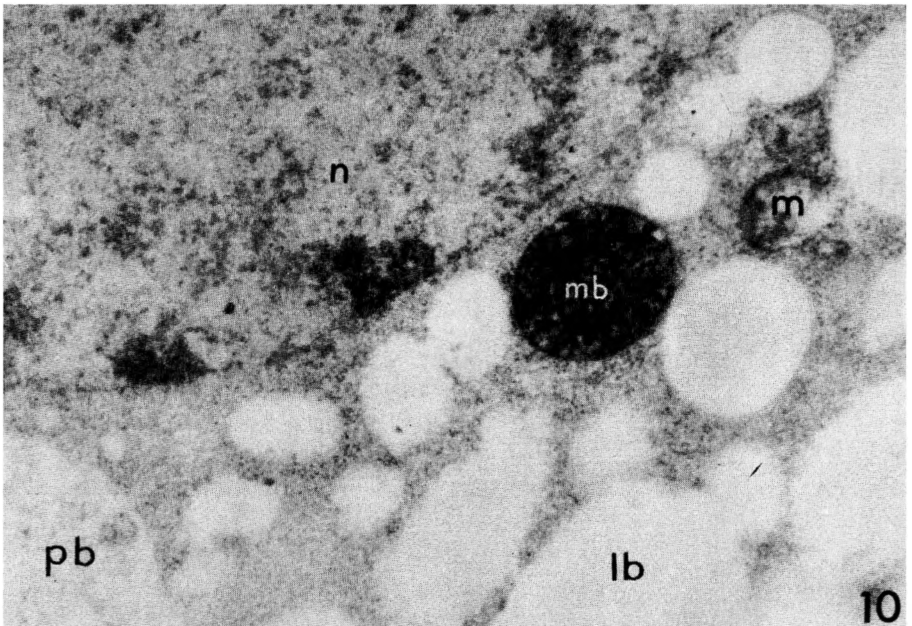


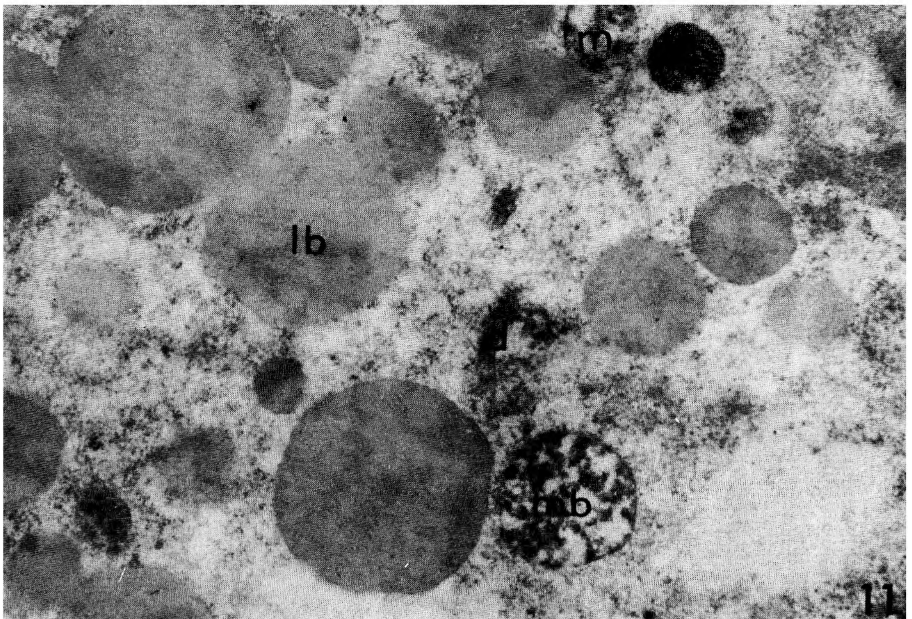
Figs. 6—7.



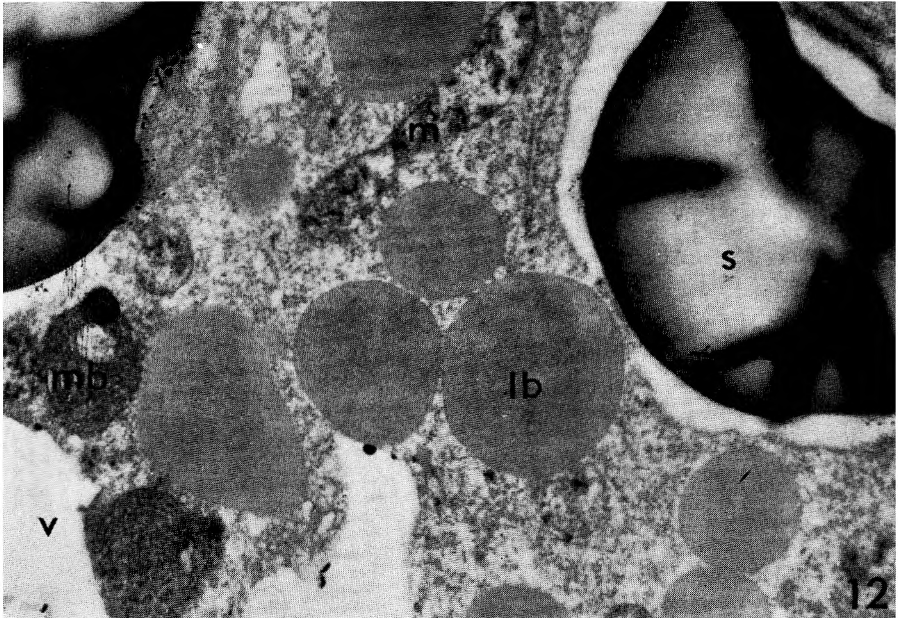


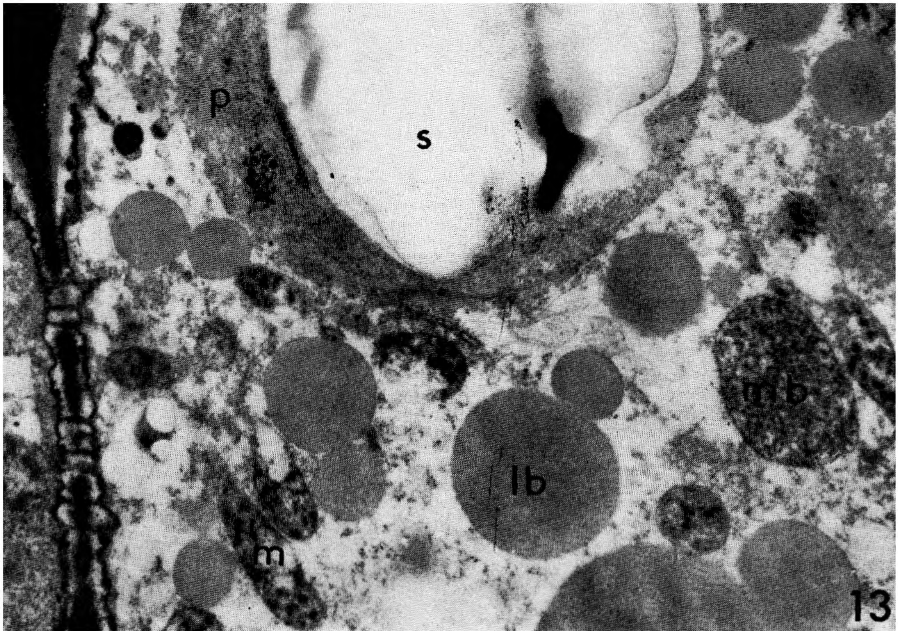
Figs. 8—9.





Figs. 10—11.





Figs. 12—13.

Isocitrate lyase. The extract of the mature silver fir seed endosperms from the Papuk region showed very low isocitrate lyase activity. It began to rise after radicle emergence and reached a maximum after 20 days' germination (Fig. 4). The endosperm extracts from the seeds of the Mašun region showed low isocitrate lyase activity throughout the experimental period. No isocitrate lyase activity was detected in the embryo of seeds from both regions.

Catalase. A considerably high activity of catalase was found in the extract of the mature seed endosperms from both the Mašun and Papuk regions (Fig. 5). In the Papuk endosperms, a peak of maximum activity was reached after 16 days' germination followed by a gradual decrease. The embryo extracts had a low catalase activity which remained the same throughout the time of the experiment in the seeds from the Mašun region. But there was no activity of this enzyme in the embryos of the Papuk seeds after radicle emergence.

Silver fir seeds showed very heterogeneous germination. The percentage of seeds reaching the same stage of development in a given time was low and it is possible that the samples collected during the early stages of germination were less representative than those where the radicle had emerged.

Electron microscopy

Segments of endosperms and embryos selected at different germination stages were examined in an electron microscope to establish the changing of microbodies *in situ*. The morphological term *microbody* is used to describe glyoxysomes, peroxisomes and nonspecialized microbodies since it is impossible to distinguish ultrastructurally between them. For the experiments we used 24 hrs imbibed seeds, seeds with a 0.2—0.4 cm long radicle and seeds with a 1.5—2 cm long radicle.

The cells of the endosperm observed 24 hrs after imbibition were packed with droplets of storage lipids (Fig. 6). Rare microbodies (presumably glyoxysomes) were squeezed among the lipid bodies and storage protein bodies. Subsequently, the number of lipid bodies decreased in the cells of the endosperm of the germinating seeds, especially in the seeds with a longer radicle, while the number of microbodies increased (Figs. 7, 8 and 9). This is the time of maximum activity of catalase and isocitrate lyase. Most of the microbodies in Figs. 8 and 9, showed, in section, invaginations and inclusions of cytoplasmic material. Such cytoplasmic invaginations and inclusions were also found in the microbodies of other germinated seeds such as cucumber, sunflower, *P. ponderosa, Pinus silvestris* (Ching 1970, Gruber et al. 1970, Trelease et al. 1971, Simola 1974).

Microbodies were also found in segments of embryos at all three germination stages although fewer in number (Figs. 10, 11, 12, 13). In embryos with a 0.2—0.4 cm long radicles they were less electron opaque and granular than at the other two germination stages.

Discussion

Our data showed that two lipases were present in the fir seeds during the period of germination, the acid and alkaline ones. The lipase activity is absent in the floating lipid layer. This is also the case with many oil seeds such as peanut, sunflower, cotton, corn and tomato (Allfrey and Northcote 1977, Huang and Moreau 1978) but not with the castor bean endosperm where the acid lipase is associated with the lipid bodies and the alkaline one with the glyoxysomes (Ory et al. 1968, Ory 1969, Muto and Beevers 1974, Huang and Moreau 1978, Moreau et al. 1980). A two-lipase system also appears in the gametophyte of Douglas fir seeds where the highest specific activity of acid and neutral lipase was found to be associated with heavy fat bodies and the soluble fraction (Ching 1968).

The lipolytic potential appeared to be high in dry fir seeds, both in the endosperm and the embryo, but little lipolysis occurred at this stage (Kovač and Vardjan 1981). This is also true with other coniferous seeds (Nyman 1965, Ching 1968). The high lipolytic activity in the dry seeds raises the problem of cellular control in preventing *in vivo* lipolysis of the storage triacylglycerols. Some authors have hypothesised the existence of a control mechanism, such as an elevation of the pH (Moreau et al. 1980) or a delay in the production of an enzyme to degrade the membrane of the spherosomes (Bewley and Black 1978). Ching's (1972) hypothesis is that the dry fatty seeds contain some soluble metabolites or hormones that are quickly produced upon hydration of the preexisting cellular constituents. Marriott and Northcote (1975) have suggested that the lipase is not in contact with its substrate or that its action is blocked by a localized inhibitor which is removed in preparation of the tissue extracts.

As the activity of alkaline lipase in the endosperm of fir seeds does not change in any appreciable way during the part of the germination period studied, we concluded that the alkaline lipase activity does not limit the mobilization of storage reserves during germination. This was also observed in the castor bean (Marriott and Northcote 1975) and ground nut (Allfrey and Northcote 1977). The activity of the acid lipase and the catalase somewhat increases after penetration of the radicle, while the activity of the isocitrate lyase shows a great increase. Their high activity is associated with the mobilization of reserve triglycerides at the time of rapid growth of the embryo (Kovač and Vardjan 1981). The lack of isocitrate lyase activity before germination seems to be an important control point for the mobilisation of oil reserves. The activity of all the enzymes assayed shows a constant decrease in the endosperm of nongerminated seeds from the Mašun region.

Lipase and catalase activity does not differ very much in the embryo of well germinated and nongerminated seeds. But their activity is almost undetectable in the well germinated seeds after radicle emergence while the activity of the nongerminated seeds remains constant (acid lipase, catalase) or shows a slight decrease (alkaline lipase). In contrast to the endosperm, the embryo of fir seeds has no isocitrate lyase present, suggesting that fatty acids are not converted to carbohydrate there. Although a small number of reports have focused on the part of the seed that does not contain reserve lipids, it seems that the absence of this enzyme is common for this tissue (Ching 1970, Tester 1976, Leung et al. 1979).

The results suggest that the activity of the enzymes assayed in the embryo are critical for the induction of germination and that the activity of the enzymes in the endosperm aids the seedling establishment after radicle protrusion.

Fine structural and cytochemical changes related to biochemical and physiological events occurring during germination were observed in the three germination stages. This study reports that microbodies are present in 24 hrs imbibed endosperm and establishes that their presence *in situ* coincides with the presence of considerable catalase activity in homogenate but with very low isocitrate lyase activity. In the other gymnosperms studied, microbodies develop at a relatively late stage of germination (Simola 1974, Simola 1976, Gori 1979). The presence of microbodies and the absence of isocitrate lyase before visible germination is also found in sunflower cotyledons (Gruber et al. 1979), cotton seeds (Bortman et al. 1981), cucumber (Köller et al. 1979) and *Pinus ponderosa* (Ching 1970). The suggestion has been made that the organelle under consideration is synthesized independently of the two glyoxysomal enzymes isocitrate lyase and malate synthetase (Gerhardt and Beevers 1970, Longo and Longo 1970). The increase in the number of glyoxysomes and the marker enzyme activity parallel the rate of lipid body disappearance in silver fir seeds with a longer radicle. The same trend of development was found in the megagametophyte of *Pinus ponderosa* (Ching 1970).

Microbodies were also present in the embryo at all the three stages examined. An absence of isocitrate lyase activity indicates a lack of glyoxylate cycle in these particles. Microbodies were occasionally also seen in the embryo of *Pinus sylvestris* and *Picea abies* (Simola 1974, Simola 1976). Ching (1970) reported some albeit negligible activity of isocitrate lyase in the embryo of *Pinus ponderosa* which indicated a lack of conversion of fats to sugars. Microbodies with only catalase, uricase and glycolate oxidase as major enzyme constituents have been isolated from a variety of other plant tissues (Huang and Beevers 1971). The authors classified them as nonspecialised microbodies.

The data presented here suggest that lipids are apparently metabolised in a different way in the embryo, where they can be reutilized within a cell, and in the endosperm where they are transported to the embryo.

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SAŽETAK

AKTIVNOST NEKIH ENZIMA LIPIDNOG METABOLIZMA U SJEMENKAMA JELE (*ABIES ALBA* MILL.) TIJEKOM KLIJANJA

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Aktivnost nekih enzima, koji u sjemenkama jele *(Abies alba* Mill.) kataliziraju razgradnju masti, tijekom klijanja u embriju i u endospermu različito varira. Mjerenja smo izvršili na sjemenkama iz Mašuna (Notranjsko, Slovenija), koje kod sobne temperature nisu klijale, i na lako klijavim sjemenkama s Papuka.

Aktivnost lipaze mjerili smo kod pH 8,5 (bazična lipaza) i kod pH 5,5 (kisela lipaza). Kisela i bazična lipaza bile su aktivne već u suhim sjemenkama obiju provenijencija. Aktivnost bazične lipaze se u endospermu prije i tijekom vidljivog klijanja nije bitno promijenila. Iz toga smo zaključile da bazična lipaza nije ograničavajući faktor klijanja. Pri prodoru korjenčića kroz testu je međutim u lako klijavim sjemenkama

aktivnost kisele lipaze i katalaze prilično porasla, pogotovu pak aktivnost izocitrat-lijaze. U endospermu sjemenki iz Mašuna, koje pri sobnoj temperaturi nisu klijale, aktivnost je svih spomenutih enzima konstantno opadala.

Kisela i bazična lipaza te kataliza bile su aktivne u embriju i prije vidljivog klijanja. Nakon prodora korjenčića aktivnost je tih enzima naglo pala, dok je kod sjemenki iz Mašuna ostala konstantna (kisela lipaza, katalaza), ili pak samo sporo padala (bazična lipaza). Izocitrat-lijaza u embriju nije bila aktivna.

Prisutnost nespecijaliziranih peroksisoma i glioksisoma utvrđivale smo elektronskim mikroskopom. Glioksisomi su bili prisutni već u endospermu suhih sjemenki. Njihov se broj povećao u sjemenkama koje su klijale. Prije i tijekom vidljivog klijanja ušle smo u trag mikrotjelešcima i u embriju. Budući da ondje izocitrat-lijaza nije bila aktivna, zaključile smo da mikrotjelešca u embriju ne djeluju kao glioksisomi. Prema svemu sudeći, razgradnja masti u embriju ne odvija se preko glioksilatnog ciklusa, kao što je to slučaj pri razgradnji masti u endospermu.

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