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The Annual Dynamics of Reserve Compounds and Hydrolitic Enzymes Activity in the Tissues of *Pinus sylvestris* L. and *Larix sibirica* Ledeb.

– The Metabolism of Reserve Compounds in the Tissues of Siberian Conifers –

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Abstract

Studies have been done on the annual dynamics of starch, low molecular carbohydrates, triacylglycerol content, amylase and lipase activities in the needles and the inner bark of 35-year-old trees of the Pinus sylvestris L. and Larix sibirica Ledeb. species from plantations near Krasnoyarsk, in the South Taiga region of Central Siberia. It was shown that triacylglycerols were the main reserve substances in the tissues of both species during most of the year. The predominance of triacylglycerols as reserve substances for the deciduous larch is less expressed than for the evergreen pine. A high concentration of triacylglycerols in storage tissues was found during the winter months. The lowest amount of triacylglycerols in the needles and the inner bark of both species was found during the periods when the temperature was near 0°C. A peak in starch levels in spring in the needles and the inner bark of the pine trees and an autumn peak in the starch levels of the inner bark of the larch trees were noted. It is concluded that starch storage in the needles and the inner bark of the pine in spring carries out the function of depositing carbohydrates originating from triacylglycerols, but in autumn the source of the starch deposits in the inner bark of the larch is a result of the carbohydrates being transported from the yellowing needles. An increase of amylase activity and a decrease of starch content in the needles and the inner bark of the studied species during the second part of the vegetation period coincided with the period of heightened intensity of cellulose being deposited into the annual wood ring. The maximum lipase activity in spring preceded the highest starch levels. It was thought that before visible growth began the energy supply function of the reserve compounds was most important, but, when the growth started – it was the substrate function.

Key words: triacylglycerol and carbohydrate annual dynamics, lipase and amylase activities, *Pinus sylvestris* L., *Larix sibirica* Ledeb.

Introduction

It is generally accepted that there is low fat content in the vegetative organs of plants (Goodwin and Mercer 1986). However, in the storage organs and tissues of woody plants, especially conifers, a rather high concentration of neutral lipids and triacylglycerols is found (Sudachkova 1977, Novitskaya 1978, Kramer and Kozlowski 1979, Rodionov *et al.* 1988). Even in the sapwood of Pinus sylvestris the triacylglycerol concentration reaches 3.5% DW (Saranpää and Piispanen 1994). Fats (triacylglycerols), along with carbohydrates, are the most important energy reserves of plants. The role of these compounds as protective substances is especially significant for evergreen conifers impacted by low winter temperatures. Triacylglycerols are accumulated in the needles and in the parenchyma elements of the wood and the inner bark (Esau 1980, Lotova 1987). Accumulation of triacylglycerols in the wood of some coniferous species has been studied in detail (Höll 1985, Fischer and Höll 1992, Saranpää and Piispanen 1994). The data for the inner bark, however, are limited (Jeremias 1969, Sudachkova 1977, Rubchevskaya and Levin 1982). An increase of fat concentration is found in tissues of many coniferous species in winter, and, in extreme temperature conditions, it is accompanied by a disappearance of starch. (Sharkov and Tsvetkova 1950, Lebedenko 1966, Karnik *et al.* 1966, Jeremias 1968, Sudachkova 1977, Rubchevskaya and Levin 1981, Rubchevskaya and Levin 1982, Repyakh *et al.* 1983).

According to the traditional division of trees into the categories of "fat-trees" and "starch-trees", the majority of conifers are related to the fat-type (Lyr *et al.* 1967). As it was shown earlier, (Sudachkova 1977), the intensity of growth of some conifer species at the beginning of the vegetation period is determined by the level of the so-called "starch maximum" which disappears by the middle of summer. Up to now the source of the starch accumulation has remained unknown. Is it newly formed photoassimilates or products of the reserve triacylglycerols breakdown? To solve this problem, one should compare the reserve polysaccharide and triacylglycerol content with the dynamics of the respective hydrolytic enzymes that destroy these compounds. Amylase and lipase activities were found in tissues of different conifer species. A breakdown of reserve triacylglycerols catalyzed by lipase has been studied in detail in germinating conifer seeds (Ching 1963, 1973, Nyman 1971, Kovač and Vardjan 1981, Kova and Wrischer 1984) and in tree

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stems in connection with the formation of the wood and the heartwood (Fischer and Höll 1992, Saranpää and Piispanen 1994, Hillinger *et al.* 1996). Lipase activity in the tissues of needles and inner bark has not been studied in depth (Sudachkova 1977). The detection of amylase in various vegetative organs of woody plants has led us to conclude that it has played a role in affecting the value and the duration of the starch maximum (Sudachkova 1977, Witt and Sauter 1994 a,b, Sauter *et al.* 1998). As to the reason why the spring starch maximum in conifers is more pronounced than in deciduous species (Fischer and Höll 1992, Piispanen and Saranpää 2001), it could be due to the fact that conifers are a more suitable subject for the study of the activity of enzymes catalyzing a breakdown of triacylglycerols and starch.

Thus, the aim of this investigation was to determine the supplies of carbohydrates and triacylglycerols and the amylase and lipase activity in the tissues of the evergreen (Pinus sylvestris L.) (pine) and deciduous (Larix sibirica Ledeb.) (larch) coniferous species.

Materials and Methods

The objects of investigation were 35-year-old Pinus sylvestris L. (pine) and Larix sibirica Ledeb. (larch) trees from the plantations in the South Taiga zone of Central Siberia near Krasnoyarsk. Beginning in April 1999, a branch was cut from the middle part of the crown of each of the 10 trees on a monthly basis throughout the year. The needles picked from each branch were mixed in order to prepare an average sample for the biochemical analysis. Pine needles were picked in April and May 1999 from the previous year's shoots and from June 1999 from the current year's shoots. Larch brachyblast needles were picked from the previous year's shoots. The inner bark samples (1x3 cm) were cut from each of the 10 trees, separated from the rough outer bark, crushed and combined to prepare an average sample. In the average samples of the needles and the inner bark the content of triacylglycerols, low molecular carbohydrates and starch were estimated.

Low molecular carbohydrates were extracted with 80% ethanol, hydrolyzed (Yermakov *et al.* 1972) and their content was determined by the discoloration of the Feling liquid (Woznesensky *et al.* 1962). Starch was extracted with perchloric acid and levels were determined iodometrically (Humphreys and Kelly 1961). Triacylglycerol content was estimated enzymatically (Kovač and Wrischer 1984). Lipase activity was estimated after the precipitation of protein by cold acetone (Scopes 1982) (Schmidt *et al.* 1974). Amylase activity was determined in purified buffering extracts (Bergmeyer 1974).

Periodically during the vegetation period, wood pieces (1x5 cm) were cut from 10 pine stems, the layer of newly formed xylem was scraped away, and the mass of absolutely dry tissue was determined gravimetrically. Cellulose content was analyzed by Kurschner's method (Obolenskaya *et al.* 1965). The results were related to 1cm^2 of cambium surface. Each experiment was repeated at least three times for each of the extracted samples. The data were presented as a mean with a standard error of the mean.

Results

Comparison of the biometric data testifies to the fact that the pine and the larch trees didn't differ much in either xylogenesis or height growth intensity. The average wood annual ring width is 2,6 mm for pine trees and 2,7 mm for larch, and the height is 15,7 m and 16,0 m accordingly. This is a reason to consider possible metabolic distinctions between pine and larch as species specific and independent of environmental conditions.

The annual dynamics of fat content in woody plant tissues is considered, as a rule, to be connected with the temperature dynamics (Sakai and Larcher 1987). Our observations were performed from April 1999 to May 2000. The duration of the frostless period in 1999 was 140 days and the duration of the period with an average daily temperature above 10°C was 129 days. The absolute annual temperature maximum was observed in July (33°C), while the absolute minimum was in January (-37°C). Dynamics of the average daily temperatures during five-day periods is presented in Fig.1.

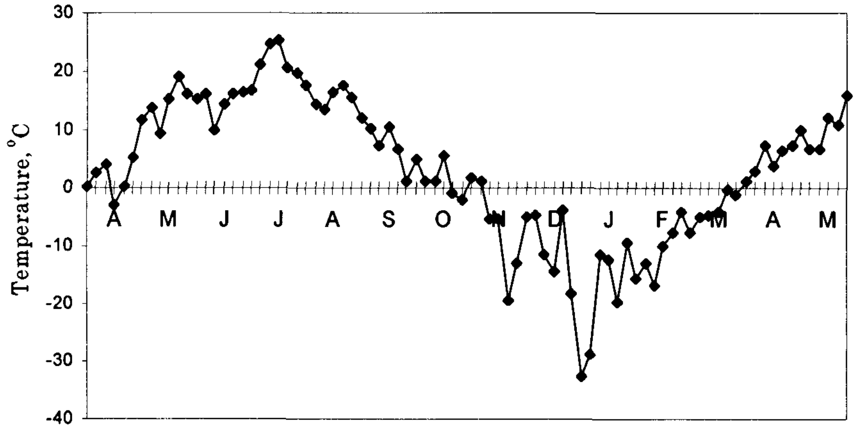

Fig. 1. Dynamics of average daily temperatures during five-day periods.

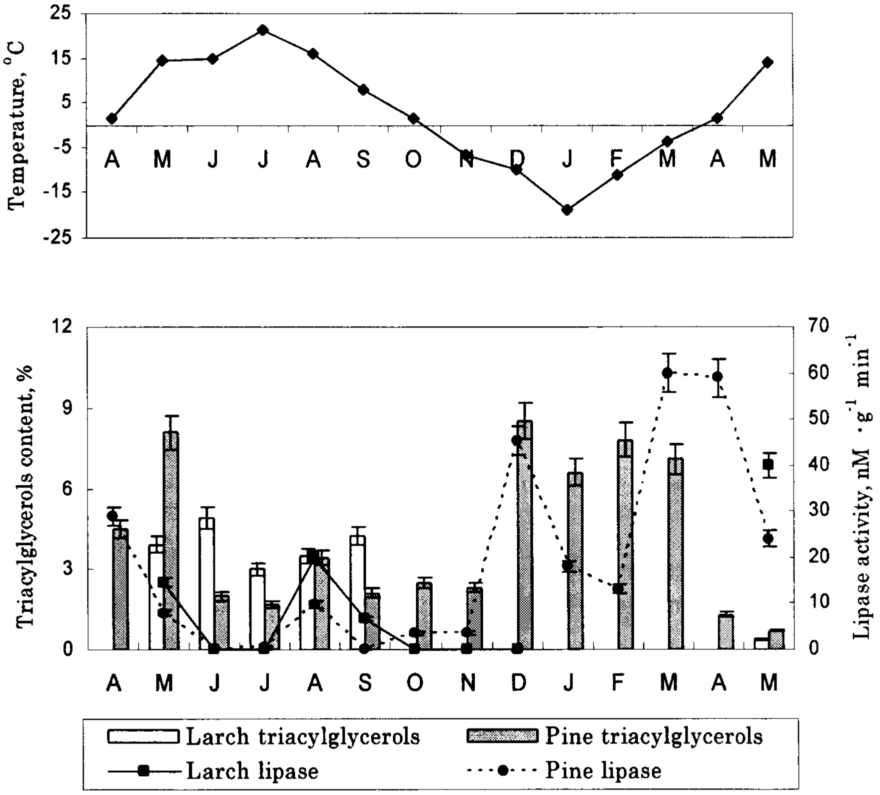

Fig. 2. Average monthly temperature and triacylglycerol content and lipase activity in the needles of pine and larch.

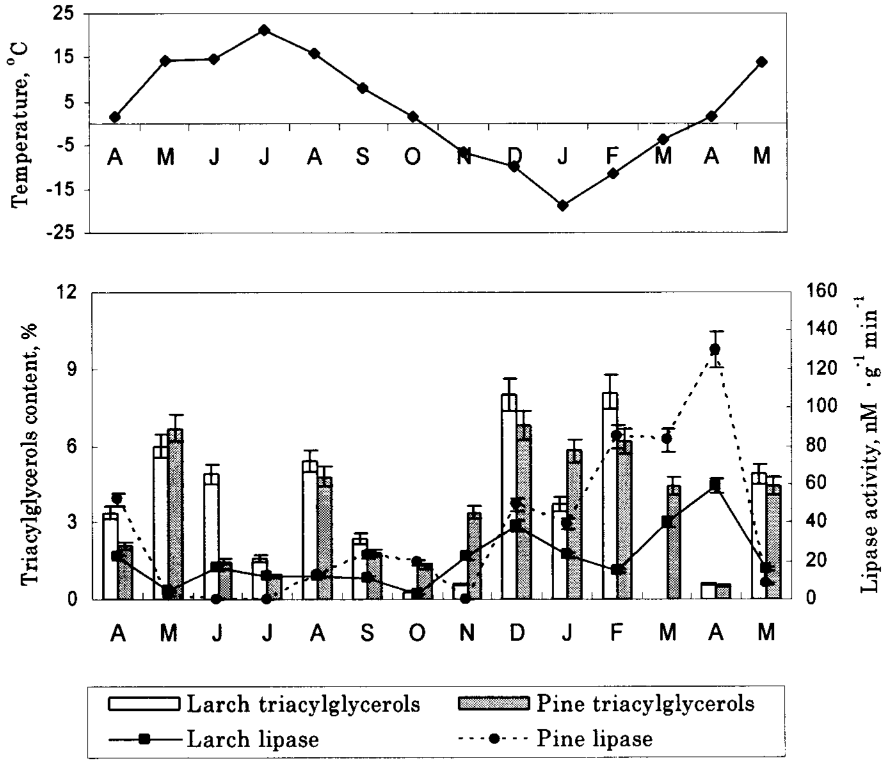

Fig. 3. Average monthly temperature and triacylglycerol content and lipase activity in the inner bark of pine and larch.

A minimal amount of triacylglycerols was found in the needles and the inner bark of both species in April and October during the period when the temperature approached 0°C (Fig.2,3). In general, the annual dynamics of the triacylglycerol content in the needles and inner bark was characterized by abrupt changes. The lowest fat content level was detected in April 1999, followed by an increase in May. During the summer and into autumn the triacylglycerols storage gradually decreased to the minimum amount seen in October-November. During the period from December to March, with average daily temperatures being lower than -10°C, the fat content sharply increased. In April it fell again but increased again in May. The fat content was very low in the previous year's pine needles during April and in newly formed larch needles in May (the month of bud breaking). In autumn, the fat content in pine needles was also low, but, in larch needles just before falling, it was rather high (4.2% DW). The early spring and autumn level of fats in the inner bark was also low. The triacylglycerol dynamics is similar for both pine and larch.

Changes in lipase activity in the pine and the larch needles differed. In the larch, high lipase activity was seen in young needles in spring and an activity increase was noted in August, while in the rest of the vegetation periods the enzyme activity was low (Fig. 2). In the pine needles, the highest levels of enzyme activity were observed in March-April. There was an increase in activity in August, a burst of it in December, and a decrease in January-February. The peaks of lipase activity were found both against the background of a high triacylglycerols concentration (December, March) and a low one (April). Lipase activity in both the inner bark and the needles rose in winter and autumn months. The activity maximum in the pine's inner bark in comparison with the needles appeared later (Fig.3). Exactly at this time in the inner bark of the pine and the larch the triacylglycerol content sharply fell.

The starch content dynamics in the pine and the larch differed greatly. From November to March, in the needles and inner bark of the pine, only traces of starch were found, (which can not be reproduced using the scale of this figure) whereas in the larch's inner bark its concentration reached 1.5-2.4% DW (Fig.4, 5).

In the pine needles the well-marked starch maximum was observed in May but in the larch needles it was insignificant at that time. The dynamics of starch accumulation in the inner bark of the larch differed from that of the pine. The autumn starch maximum reaching 13% DW was observed in the larch's inner bark but it was not detected in pine. This seems to be a result of a starch breakdown and an intensification of the flow of basipetal low molecular carbohydrates from the yellowing needles and their temporary deposit into the inner bark tissues.

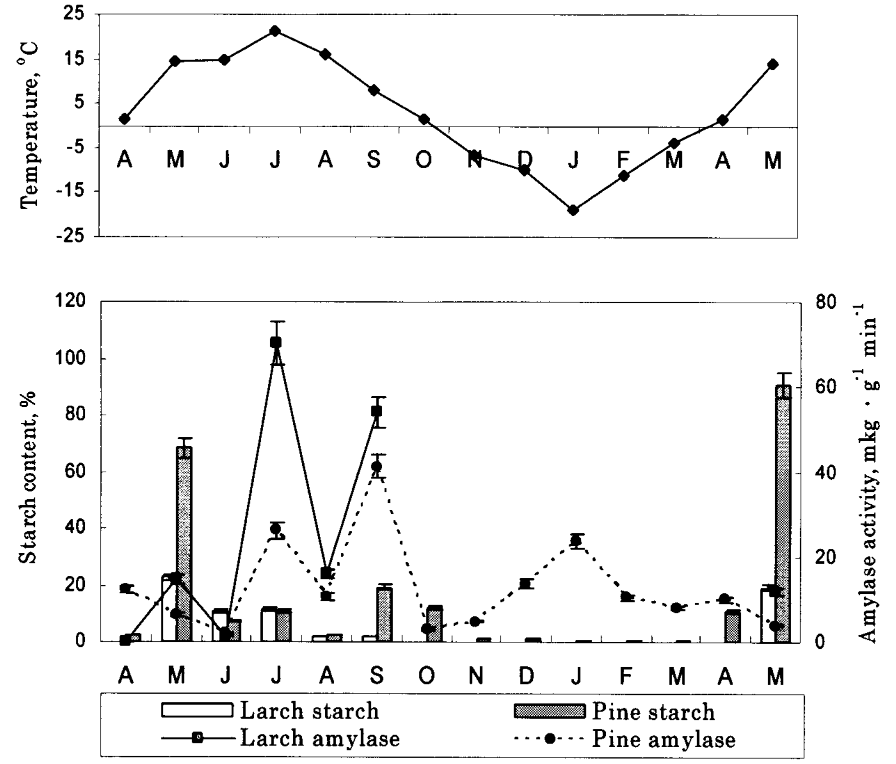

Fig. 4. Average monthly temperature and starch content and amylase activity in the needles of pine and larch.

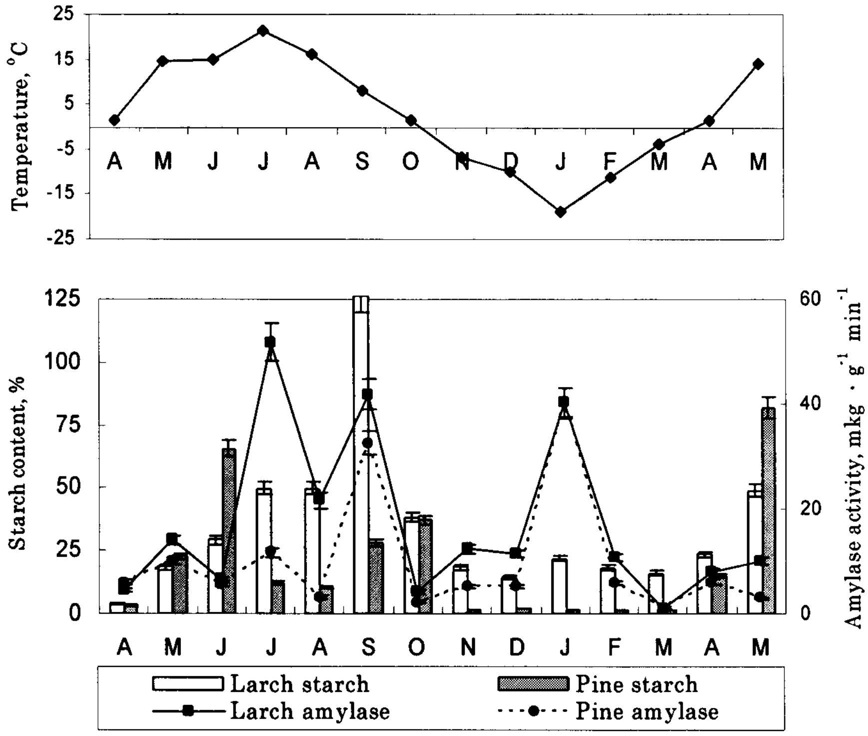

Fig. 5. Average monthly temperature and starch content and amylase activity in the inner bark of pine and larch.

Amylase activity in the needles of both the larch and the pine had clear peaks in July and September. The May starch maximums coincided with the lowered amylase activity (Fig.4). This seems to be a result of a starch breakdown and an intensification of the basipetal flow of low molecular carbohydrates from the yellowing needles and their temporary deposit into the inner bark tissues.

An increase of amylase activity in the inner bark and the pine needles of the larch was found in July, September and January. A spring starch maximum in the pine's inner bark (May-June) was found during the period of the low amylase activity both in 1999 and 2000. Summer and autumn starch accumulation in the inner bark of the larch took place with a high amylase activity (Fig. 5). An increase of amylase activity in the inner bark of the larch and the pine needles was found in July, September and January.

Low molecular carbohydrates content in the needles of pine and larch, as a rule, did not exceed 5% DW (Fig.6). In the inner bark of both species the low molecular carbohydrate concentration was usually more than in the needles, and in some cases (in April and November in the larch inner bark) reached 10% DW. These periods coincided with the lowest content of reserve carbohydrates and fat compounds (Fig 2-5). These peaks appeared to be a result of two processes - starch and fat destruction. As a result, the low molecular carbohydrate pool got re-stocked both by glucose from starch and by products of the transformation of glycerol and fatty acids originating from fat in the gluconeogenesis process.

In order to best illustrate the role of two basic reserve stocks — triacylglycerols and starch in the pine and the larch's annual cycles, the mass ratio of these compounds is shown (Tab.).

It is obvious that in the pine needles and the inner bark the exchange of fat reserve compounds for carbohydrates took place in April, and the reverse process took place in November-December. It is notable that this process is likely to be easily reversible. In August a sharp increase of fat content was observed and then in September it fell sharply. In the inner bark of the larch, such a clear exchange of triacylglycerols for starch was not observed, but the tendency still persisted. In the larch needles, by the end of vegetation, starch almost disappeared and triacylglycerol content was sufficiently high, which manifested itself in the relative index growth. It is necessary to note that the maximal index values for the larch's inner bark were lower compared to those for the pine.

Earlier it was shown that the intensity of reserving was linked with the intensity of the growth processes. The decrease of the triacylglycerols concentration in tissues preceded the growth of shoots (Sudachkova 1977). The main mass of assimilates in woody plants was used for wood formation during the process of xylogenesis. The xylogenesis intensity was characterized by the accumulation of wood mass in the pine's annual ring, counted by the units of cambial surface producing tracheids (Fig. 7).

During the first month of the vegetation period the

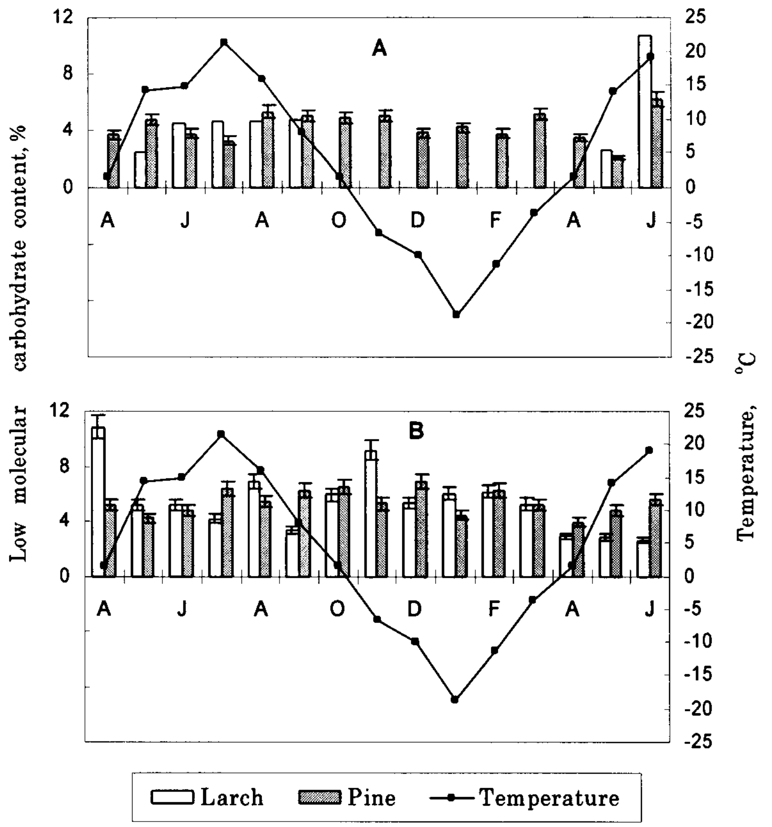

Fig. 6. Average monthly temperature and low molecular carbohydrate content in the needles (A) and inner bark (B) of pine and larch.

Table. Mass ratio of triacylglycerols: starch in pine and larch tissues.

Month	Larch needles	Pine needles	Larch inner bark	Pine inner bark
April		17.3	8.3	6.4
May	1.7	1.2	3.3	2.9
June	4.5	2.8	1.7	0.2
July	2.7	1.6	0.3	0.8
August	18.4	13.6	1.1	4.7
September	22.1	1.1	0.2	0.6
October		2.0	0.1	0.3
November		17.7	0.3	26.2
December		85.0	5.6	30.9
January		82.5	1.7	38.7
February		111.4	4.5	38.8
March		78.9	0.0	29.3
April		1.2	0.3	0.3
May	0.2	0.1	1.0	0.5

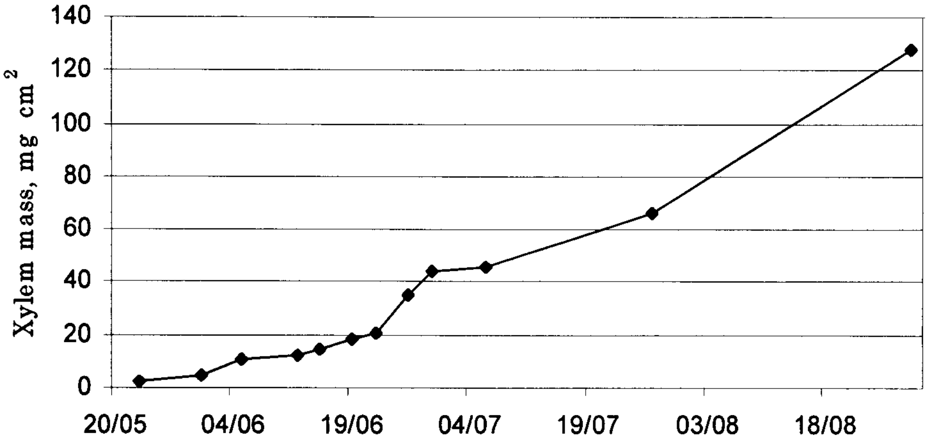

Fig. 7. Dynamics of pine wood mass increasing on the unit of stem surface.

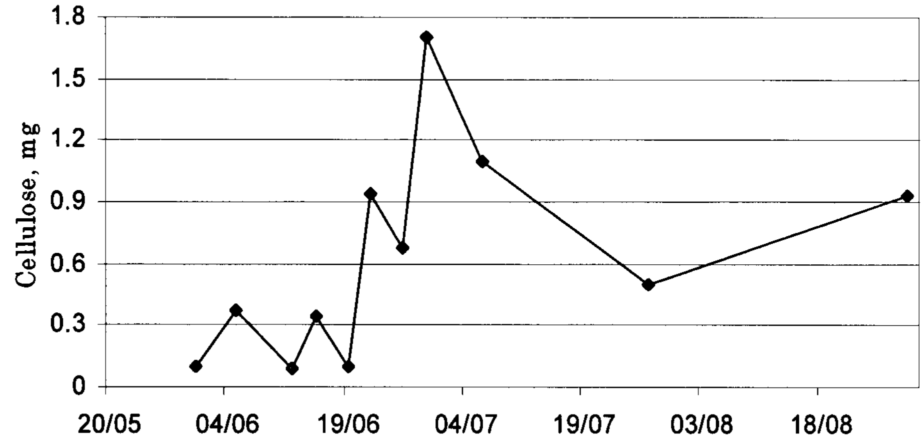

Fig. 8. Intensity of cellulose synthesis in pine wood on the unit of stem surface.

intensity of the biomass increase was insignificant. It corresponded to the rate of increase of the tracheid amount in the annual wood ring. At the end of July the rate of biomass accumulation grew sharply. It coincided with the start of the late wood formation (Sudachkova *et al.* 1973, Antonova 1999). From this moment on, the growth in wood mass was not so much the result of the increase in the tracheid number in the annual ring, but of the increase in the width of the late wood cell walls. Determining the cellulose content in wood samples allowed us to account for the cellulose increment, per unit of the cambial surface in a twenty-four-hour period, based upon the above-mentioned data of wood mass growth. The intensity of cellulose synthesis per twenty-four-hour period reached the maximum at the end of June, dropped in July, and increased again in August during the period of the formation of the cell wall tracheids of the late wood (Fig. 8). Thus, it was during the second part of the vegetation period that the formation of the basic mass of the annual wood ring occurred.

Discussion

Seasonal changes in the content of the reserve substances in the vegetative organs and tissues of woody plants have been studied for more than 100 years (Faminzin and Borodin 1867, Russov 1882). As of yet, no consensus has been reached regarding the reasons for this phenomenon because the dynamics of seasonal changes differ depending on the species and the habitat. For example, during the vegetation period some investigators observed only a single starch peak in tissues of woody plants in the spring (Novitskaya 1971, Sudachkova 1977), but the others noted this peak in both spring and autumn, or only in autumn (Kozlowski and Keller 1966, Piispanen and Saranpää

2001). The data concerning the seasonal dynamics of triacylglycerols are also contradictory. It was found that the amount of triacylglycerols in the sapwood of the Scotch pine did not change throughout the entire year (Saranpää and Nyberg 1987, Fischer and Höll 1992), but in the poplar wood it increased in spring and autumn (Höll 1985, Sauter and van Cleve, 1994).

Analysis of the obtained data showed that the reserve fat and carbohydrate compounds were present in the needles and the inner bark of both the pine and the larch during the annual cycle in the form of starch and triacylglycerols. The ratio of these compounds differed considerably in the summer and winter periods. Some differences were observed between the pine and the larch in the reserve compounds localization and the preferential use of fat and carbohydrate reserves. The studied species differed in the starch dynamics. Notably large differences were found in the needles. It was caused by the differences between the needle functions of the deciduous larch and the evergreen pine. The latter used needles as a reserve organ that resulted in the appearance of the marked spring starch maximum in the pine needles. Larch differed from pine by the presence of the marked autumn starch maximum in the inner bark. The triacylglycerol content in both the pine and the larch needles during the vegetation period was higher than the starch content (with the exception of the spring maximum in 2000). In the inner bark the fat reserves were less significant and had two maximums of triacylglycerol accumulation in winter and in autumn. As we showed earlier, the intensity of the storage was linked with the intensity of the growing processes. The lowest level of the triacylglycerol concentration in the spring preceded the beginning of the shoot growth (Sudachkova 1977). Judging by the dynamics of the pine wood mass increment the July minimum of triacylglycerol content was connected with the increase of consumption for xylogenesis. The minimum of the triacylglycerol content under temperatures close to 0°C may be connected with the processes of acclimation to low temperatures when the change of plasmatic membrane structure occurred (Yoshida 1984, Fujikawa 1994).

Lipase is responsible for the processes of triacylglycerol degradation in the storage tissues. The breakdown of these compounds in the vegetative organs of the woody plants was studied more intensively in connection with the heartwood formation (Hillinger *et al.* 1996). It was shown that only a small part of energy of the triacylglycerol molecule belonging to the glycerol was used for the synthesis of secondary substances of heartwood, but the released fatty acids remained unutilized (Saranpää and Piispanen 1994). According to our investigations, the lipase dynamics in the pine needles had a strongly pronounced maximum in the spring months, preceding the start of the vegetation period, and in December, with the minimum occurring in summer and autumn. The increase of lipase activity in December was, possibly, related to the increase of glycerol concentration that was released from the fats and acted as a cryoprotector (Sauter and van Cleve 1994).

Participation of amylase in the starch breakdown in the tissues of the woody plants is discussed mostly in connection with seed germination, wood formation, cold acclimation and tolerance to environmental stresses (Nyman 1971, Sudachkova *et al.* 1981, Sudachkova *et al.* 1993, Witt and Sauter 1994b). However, the role of this enzyme in seasonal starch dynamics remains obscure. According to our data, amylase activity was low in the periods of the starch maximums, except during the autumn starch peak in the inner bark of the larch that coincided with the high amylase activity. Similar dynamics of amylase activity were observed in the poplar wood. Enzyme activity was high in March and April and after that it decreased in May and June during the time of spring starches being deposited (Witt and Sauter 1994b).

The origin of the autumn peak of the starch content in contrast to the spring one was not related to the heightened photosynthesis intensity, but with the basipetal transport of the low molecular carbohydrates from the yellowing needles. The high level of amylase activity in this period stimulated a transformation of the reserve form of carbohydrates into the transport form and a basipetal flow from stem to roots. It was shown for the deciduous trees that the starch degradation in autumn months was controlled by starch grain bound endoamylase, which has an increase in activity under the impact of low temperatures (Witt and Sauter 1994a, Sauter *et al.* 1998). Thus, in autumn the inner bark of the larch as a deciduous species performed a function of acting as a temporary depository for non-structural carbohydrates. Evidently, the source of the monosaccharides for the spring starch peak in the inner bark was fats, but for the autumn peak it was the monosaccharides being transported from the yellowing needles. Since our investigation only tested total amylase activity, it is possible that the spring and autumn breakdown of starch in conifer tissues was also catalysed by different enzymes, but this possibility requires further investigation.

On the whole, the triacylglycerol and starch dynamics in the inner bark and the needles during the test period did not reveal a uniform dependence on the activity level of the respective hydrolitic enzymes. Nevertheless, the maximum values of lipase activity and the minimum values of amylase in March and April allowed us to conclude that these enzymes were active in the spring starch maximum formation. It is possible that glycerol and fatty acids, released from fats due to lipase activity, turned into glucose during the gluconeogenesis process. Then the glucose was mixed into the starch, which accumulated in this period while being assisted by the low level of amylase activity. The increase of amylase activity and the decrease of starch content in the pine and larch needles and the inner bark during the second part of the vegetation period coincided with the period of heightened intensity of cellulose being deposited into the annual wood ring.

The need of the reserve compounds for conifers was strongest at the beginning of vegetation, when the apical and lateral meristems began to function at the same time (they provided height and diameter growth), as well as at the end of vegetation, when the process of xylogenesis had ended and pre-conditions for a successful passing and finishing of the dormancy period were created. Then the glucose was included into the starch, which accumulated in this period while being assisted by the low level of amylase activity. In addition, the deciduous larch needed reserve compounds for brachyblast needle formation in the spring. During the different periods of the annual cycle, the substrate, or energy functions, of the reserve compounds became more pronounced. As a source of energy the fats were the preferred compounds, as demonstrate by the fact that the amount of energy released per unit of mass was more than twice as much for fats as for carbohydrates. As a substrate reserve, starch was most important because it could be easily mobilized for the formation of cellulose — the main material of the cell walls in the growing needles, shoots and stems. In spring, before the beginning of growth, the respiration became more active, and fatty acids released as the result of lipase activity were the energy-rich substrate for this process. During the different periods of the annual cycle, the substrate or energy functions of the reserve compounds became more pronounced.

Thus, the obtained data allows us to conclude that the fat breakdown by lipase was more active before the beginning of the growth processes. At the next stage, during the period in which the shoots begin to grow, the cambial activity, and the needles begin to unfold, it was more important to have substrate reserves, as demonstrated by the appearance of the spring starch maximums. Fats were a deeper level of energy reserve in comparison with starch. Therefore, in the period preceding active xylogenesis, the temporary accumulation of the surplus metabolites took place in the form of starch storage, which is a concentration of glucose residues that are needed for the synthesis of the main wood component — cellulose. A more complete picture of fat and carbohydrate reserves interconversion can be obtained by comparing the dynamics of the catabolic and synthesising enzymes activities. At the next stage, during the period of the cambial activity in which the shoots begin to grow and the needles begin to unfold, it was more important to have substrate reserves, as emonstrated by the appearance of the spring starch maximums.

Acknowledgements

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