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SEASONAL CHANGES IN CHLOROPLASTS OF BLACKBERRY LEAVES

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The ultrastructure, pigment content and photosynthetic activity of chloroplasts in blackberry (*Rubus fruticosus* L. s. l.) leaves were investigated during spring, summer, autumn and winter. The fine structure of young spring leaves shows all characteristics of the young leaf tissue with the maximum value of photosynthetic activity. Their ultrastructure changes parallelly with the development and maturation of these leaves through summer and autumn. Chloroplasts of the summer leaves have a well developed thylakoid system with big grana, and starch is always present in the stroma. The content of pigments reaches its maximum in the summer leaves but the photosynthetic activity slowly falls. A reduction in the thylakoid system and enlargement of plastoglobules are the features of chloroplasts in the autumn leaves. Their photosynthetic activity and content of pigments are low, so these leaves gradually decay. New leaves which grow up in the late summer and early autumn, remain on the shrubs throughout winter, and end their lifespan in the late spring. Chloroplasts of the winter leaves have a well developed thylakoid system and numerous plastoglobules in the abundant stroma. It is significant that these leaves are exposed to low temperatures and short days throughout the period from autumn to winter and thus they are adapted to low temperatures. The winter leaves have large chloroplasts and a high content of pigments, but their photosynthetic activity is relatively low. The fine structure of the winter leaves frozen to subzero temperatures (up to —12°C), was also examined. Cells of these leaves have a dense cytoplasm pushed among cell organelles, many small vacuoles and numerous vesicles.

Introduction

Blackberry shurbs retain some leaves throughout the year. Young leaves that sprout during spring and summer, finish their lifespan in the late autumn, whereas those that grow up during the late summer and the early autumn survive the winter. In winter their senescence is slowed down and they end their lifespan in the late spring.

In the period from autumn to winter these leaves are exposed to low temperatures and short days. Continuous fall of the temperature and the change of photoperiods are only two factors that have an influence on blackberry plants and cause their cold hardening (Burke et al. 1976, Steponkus and Lanphear 1968, Huner 1986). Hormonal changes are supposed to be the cause of this process (Lalk and Dörffling 1985), since they underlie series of biochemical and physiological changes in the cells of overwintering leaves. Considerable increase of lipids (Senser and Beck 1984, Havaux et al. 1984), water-soluble proteins, free sugars and some other cell constituents (Parker 1962) have already been described. Some of them cause a higher osmotic value of the cell, which is one of the mechanisms allowing survival at low temperatures (Olien 1967, Williams and Hope 1981).

Blackberry leaves show typical differences with respect to the changes in the membrane lipid composition and the chloroplast ultrastructure (Kunst 1986, personal communication, Wrischer and Modrušan 1985). Those differences are correlated with the degree of frost resistance achieved during the adaptation to subzero temperatures. According to Levitt's classification (1980, cit. Senser and Beck 1984) blackberry belongs to the group of »very hardy« plants which are resistant to temperatures as low as — 20 °C, but they can't survive lower temperatures. Some other plants have similar temperature minima, as for example ivy (Senser and Beck 1984) and strawberry (O'Neill et al. 1981).

This study was undertaken in order to obtain new data about the ultrastructure of chloroplasts in overwintering blackberry leaves which are adapted to low temperatures and find out whether there are some seasonal differences in ultrastructure. In addition to that, the concentration of pigments and photosynthetic activity of these leaves were examined and compared.

Material and Methods

The experiments were carried out on leaves of blackberry shrubs (*Rubus fruticosus* L. s. l.) growing in the garden of the Rudjer Bošković Institute, Zagreb. Leaves were examined during spring, summer, autumn and winter for a period of two years.

For the electron microscopic examinations small pieces of leaves were fixed in 1% glutaraldehyde in cacodylate buffer (pH 7.2) with addition of 1% caffeine (Vaughn and Wilson 1981). Winter leaves frozen outdoors to temperatures from 0 °C to — 4 °C were fixed at the same temperatures below zero without any addition into the fixative. Fixation of leaves frozen to lower temperatures (—4 °C to —12 °C) needed the addition of supstances that lower the freezing point of the liquids. For this purpose ethylene glycol (5%) or sodium chloride (0.5 and 1 mol) were used. Preliminary experiments showed that the addition of these

supstances did not cause any visible ultrastructural changes. After the fixation, part of the material — used later for morphometric measurements — was postfixed in 5% $KMnO_4$ and the other in 1% OsO_4. After the dehydration the material was embedded in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds 1963) and examined in a Siemens Elmiskop I. Sections of the leaf material embedded in Araldite (about 0.5 μm thick) stained with toluidine blue were also examined in the light microscope.

Morphometric measurements were performed on micrographs (of 30,000 times final magnification) on which a 5 mm square lattice was additionally copied. The area of plastid section surface occupied by different plastid components was estimated from the number of hits which the crossings of the lattice, covering a certain plastid structure, formed (Wrischer et al. 1976, Williams 1977).

The pigments were extracted in 80% acetone. Chlorophylls were quantitatively determined according to Holden (1965), and the total carotenoids according to Urbach et al. (1976).

Photosynthetic activity of leaf pieces was measured with an O_2 electrode (Hansatech Ltd., England). The reaction mixture contained 0.1 mol phosphate buffer (pH 7.2—7.4) and 0.1 mol sodium bicarbonate (Miles 1980). The samples were illuminated at saturating illumination with a halogen lamp giving an intensity of 55×10^3 lx.

Results

The ultrastructure of chloroplasts in blackberry leaves in the period from spring to autumn shows changes typical of normal development and senescence of the leaves. Similar changes happen in the chloroplasts of overwintering leaves in the period from the late summer till spring, but senescence of these leaves is slowed down during the winter.

The light microscopic observations show that the summer leaves are thicker ($\sim$ 11 μm) than the winter ones ($\sim$ 8 μm) due to larger cells. There are no other considerable differences in their anatomy; both have two cell layers in the pallisade and in the spongy mesophyll. There are some more chloroplasts across a leaf section in the summer leaves than in the winter ones.

Young spring leaves have a typical ultrastructure. The cells contain several small vacuoles and their cytoplasm is rich in ribosomes. Chloroplasts are often found in the stage of division. Their grana are built up from 2—7 thylakoids and they are mutually connected with stroma thylakoids which stretch along the chloroplasts. The stroma has many ribosomes and only a few starch grains (Fig. 2).

Summer leaf cells (in July and August) have a single large central vacuole, while the nucleus and all other cell organelles lie peripherally. The elongated chloroplasts have a well developed thylakoid system which, according to morphometric measurements, occupies 49.5% of the total plastid section surface (Fig. 1, Table 1). Grana are very large (12—40 thylakoids per granum). The frequently observed negative contrast of thylakoids and the electron optically dense stroma originate from phenols that flow out from the vacuoles during the fixation with glutaraldehyde. While there are always starch grains in the stroma, plastoglobules are not numerous (Fig. 1, Table 1).

Table 1. The percentage of total plastid section surface contributed by different plastid components.

	Summer leaves	Autumn leaves	Winter leaves	Overwintered leaves
All thylakoids	49.5%	49.0%	45.7%	43.1%
Grana thylakoids	35.1%	32.6%	26.8%	26.9%
Starch	12.9%	10.4%		12.2%
Plastoglobules	2.5%	0.6%	4.4%	4.7%
Stroma	35.1%	40.0%	49.9%	40.0%

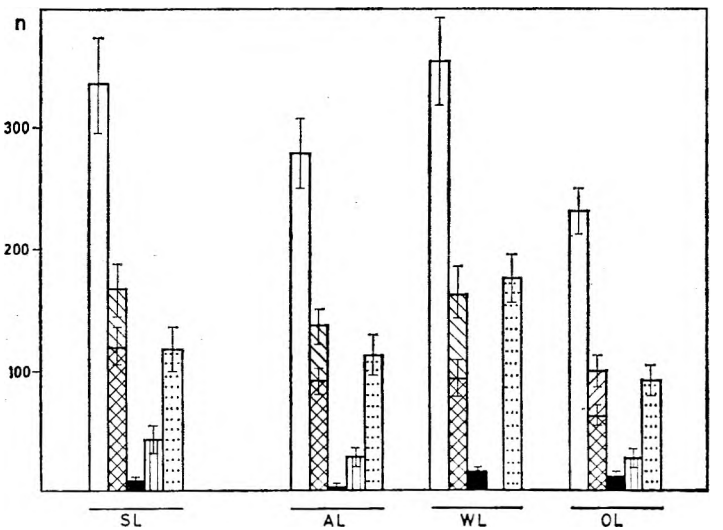
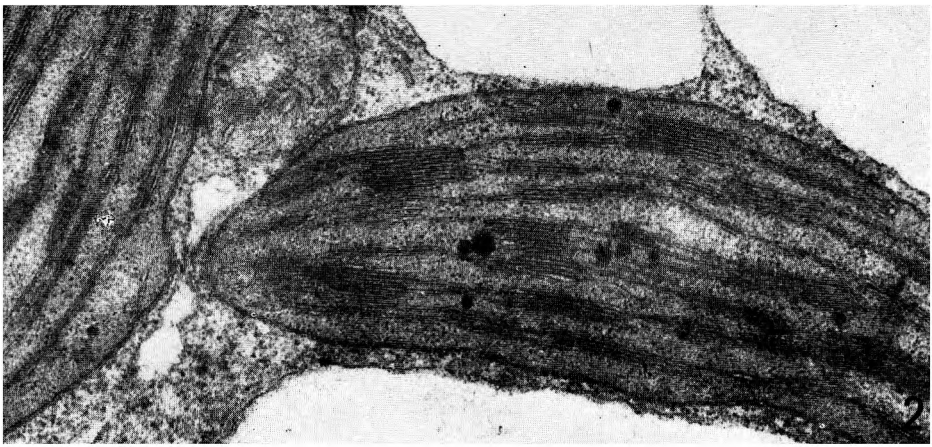
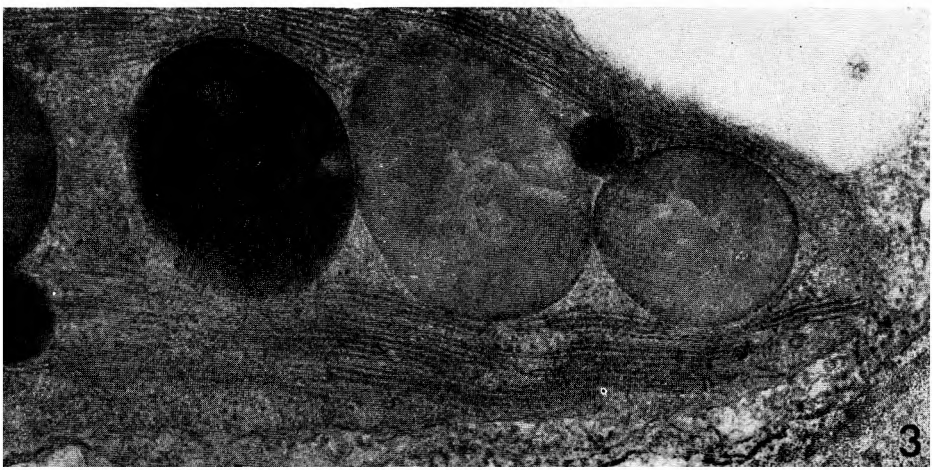
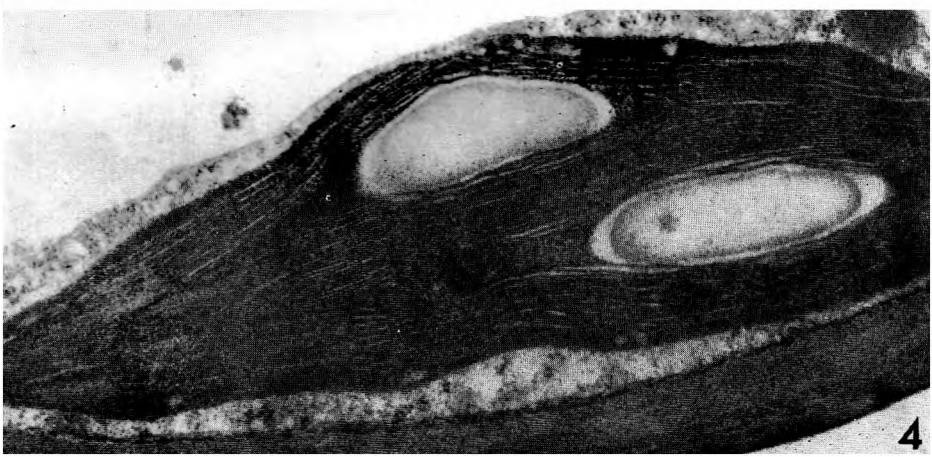


Fig. 1. The number of hits (mean value with standard error indicated) on plastid components counted by crossings forming a lattice of 5 mm squares overlying micrographs (at final magnification of 30,000 times). SL = summer leaf; AL = autumn leaf; WL = winter leaf; OL = = overwintered leaf; n = number of hits; white columns = plastids; columns with diagonal lines = all thylakoids; columns with crossed lines = grana thylakoids; black columns = plastoglobules; columns with vertical lines = starch; columns with dots = stroma.

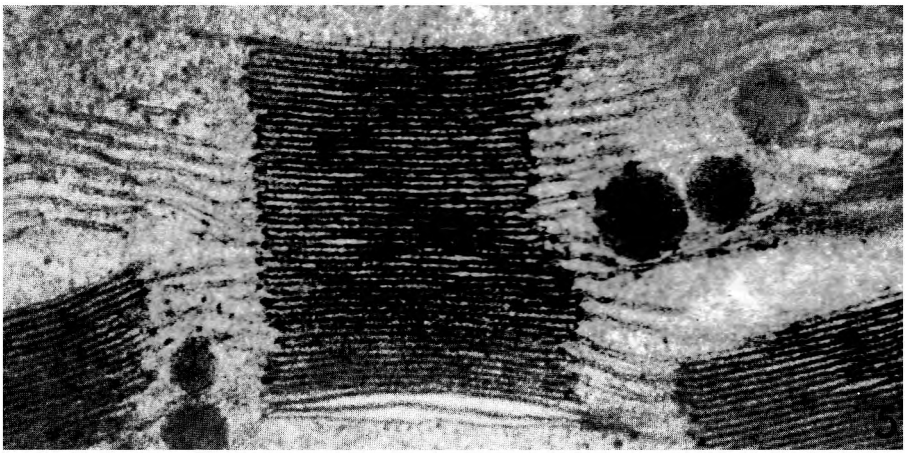
Fig. 2. Chloroplast from the young spring leaf. 36,000 : 1.
Fig. 3. Portion of a chloroplast with large plastoglobules from the yellow autumn leaf. 54,000 : 1.
Fig. 4. Chloroplast from the young autumn leaf. 30,000 : 1.
Fig. 5. Portion of a chloroplast with large grana from the winter leaf. 70,00 : 1.
Fig. 6. Cell from the frozen winter leaf (—3°C). 11,000 : 1.

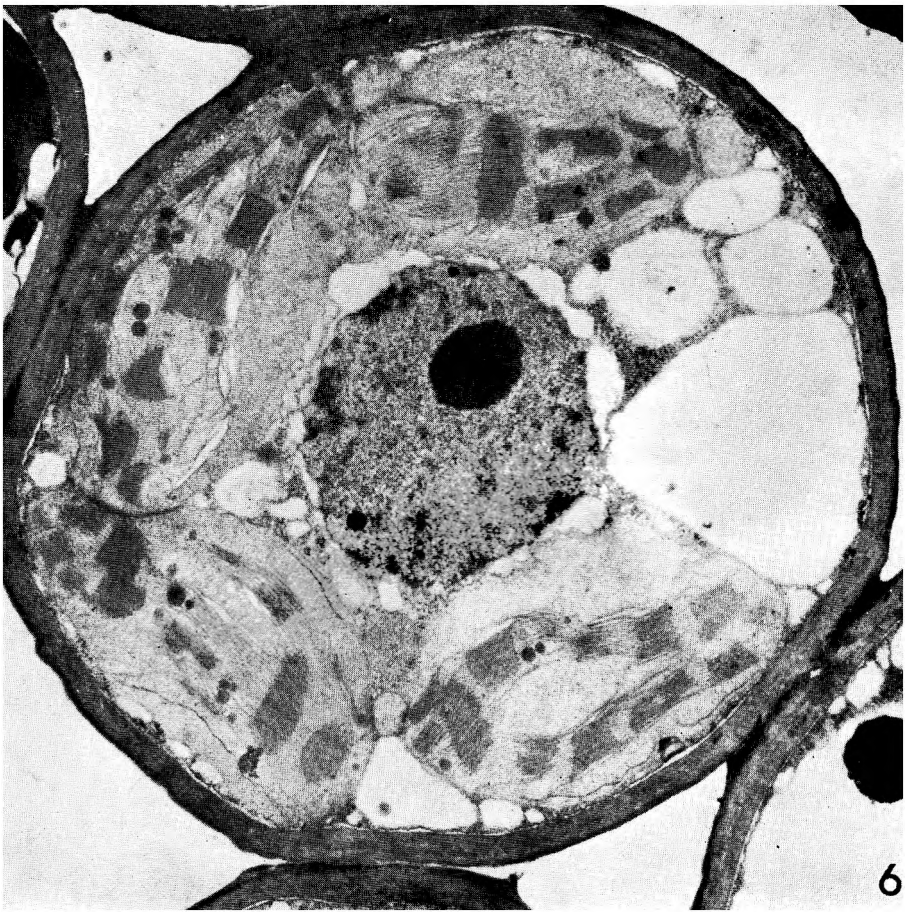






Figs. 2—4.





Figs. 5—6.

The ultrastructure of leaves shows that they gradually senesce and decay in autumn. The thylakoid system of the chloroplasts is strongly reduced, but the number and size of plastoglobules are considerably increased (Fig. 3). In the stroma there are crystalloids or clusters of dark particles about 6 nm in diameter. It could be concluded that because of their morphology they represent phytoferritin (L j u b e š i ć 1976). Leaves with such structural characteristics finish their lifespan in the late October or November.

The ultrastructure of young autumn leaves is similar to that of the summer leaves. Chloroplasts are elongated and they possess a well developed thylakoid system which occupies 49% of the total plastid section surface (Fig. 1, Table 1). The number of grana, as well as the number of thylakoids per granum, is slightly smaller than in chloroplasts of the summer leaves. Negatively contrasted thylakoids often appear. There are always some starch grains and plastoglobules in the stroma (Fig. 4)

The main characteristics of the winter leaf cells are the regular presence of several small vacuoles and a well preserved cytoplasm. Chloroplasts are larger than those in the cells of spring, summer or autumn leaves (Fig. 1). Their well developed thylakoid system makes 45.7% of the total plastid section surface. Grana are very large and built up from 22—50 thylakoids (Fig. 5). They occupy 26.8% of the total plastid section surface. These winter chloroplasts contain an abundant stroma, which makes half of the total plastid section surface (49.9%). Plastoglobules are numerous, but starch is regularly absent (Table 1, Fig. 1). Long membranes in the stroma spread parallelly with the chloroplast envelope or, when forming circular structures, they are part of the peripheral reticulum.

The fine structure of the winter leaves which have been frozen outdoors to temperatures below zero (up to — 12 °C) is similar to that of the unfrozen ones. The cell organelles are often aggregated and pulled into one part of the cell. The protoplasts are sometimes slightly detached from the cell walls. Numerous small vesicles in the cytoplasm are part of the delated endoplasmic reticulum or the nuclear envelope. The cytoplasm is dense and pushed among other cell organelles. Oval chloroplasts with an abundant stroma and large grana are always well visible (Fig. 6).

In spring the cells of overwintered leaves again contain elongated chloroplasts which are much smaller than those of the winter leaves. Their thylakoid system is reduced and occupies 43.1% of the total plastid section surface; grana thylakoids make 62.4% of that. The number of thylakoids per granum varies from 10—30. Starch grains are numerous, as are large plastoglobules (Fig. 1, Table 1).

Table 2 shows that the concentration of the total chlorophyll rises in the period from spring till summer when it reaches 3.33 mg/g of fresh leaf weight. During autumn the chlorophyll content falls to a minimum in November of 0.58 mg/g fresh weight of yellow leaves. This change in the concentration of chlorophyll corresponds to the period of senescence of the leaves. Young autumn leaves have a similar chlorophyll content as young leaves in spring. During winter the concentration of the total chlorophyll rises considerably (4.50 mg/g fresh weight), but in spring in overwintered leaves it falls slightly. Similar changes can also be noticed when the content of carotenoids is observed (Table 2).

The photosynthetic activity has the highest value in young spring or young autumn leaves (Table 2). The activity falls during summer and

Table 2. Total chlorophyll, total carotenoids and photosynthetic activity in blackberry leaves.

		Total chlorophyll (mg/g fr. wt.)	Total carotenoids (mg/g fr. wt.)	Photosynthetic activity (μmol O_2/g fr. wt./h)	Photosynthetic efficiency (μ mol O_2/mg chlorophyll/h)
SP	Young spring leaf	2.71	1.05	324.0	119.6
	Grown up spring leaf	3.32	1.19	257.1	77.4
SU	Summer leaf	3.33	1.15	195.0	58.6
AU	Green autumn leaf	2.72	1.09	182.5	67.1
	Yellow autumn leaf	0.58	0.35	52.5	90.5
	Young autumn leaf	2.95	1.05	315.0	106.8
WI	Winter leaf	4.50	1.70	185.0	41.1
SP	Overwintered leaf	3.72	1.31	285.0	76.6

SP = spring; SU = summer; AU = autumn; WI = winter.

winter when leaves get older. In spring the overwintered leaves again have a high value of photosynthetic activity (285.0 μmol O_2/g fresh leaf weight/h). The photosynthetic efficiency depends on the chlorophyll content of the chloroplasts and is very low in winter leaves (Table 2).

Discussion

The lifespan of blackberry leaves is seasonally dependent. The leaves which sprout in spring or early summer senesce and decay in autumn. Their senescence is characterized by progressive degradation of chloroplast thylakoids and by a simultaneous accumulation of plastoglobules and phytoferritin in the stroma (Ljubešić 1976). On the other hand, the leaves which grow up in the late summer or the early autumn, remain on the shrubs throughout the winter and finish their lifespan in the late spring. During autumn and winter those leaves undergo the process of cold hardening when low temperatures and probably low illumination conditions play an important role (Steponkus 1981, Huner 1986).

Morphometric analysis has shown that there are significant differences among the chloroplasts of autumn, winter and overwintered leaves. The chloroplasts of the winter leaves are very large due to an abundant stroma. Their thylakoid system, although well developed, is quantitatively very similar to that in the summer chloroplasts. In both cases chloroplasts contain large grana, which is a feature of grown up chloroplasts. The abundant stroma seems to be a characteristic of the winter chloroplasts. In the overwintered chloroplasts stroma is gradually reduced in spring together with the thylakoid system. The curious augmentation of the peripheral reticulum, found in the winter blackberry chloroplasts, was also noticed in other cold hardened leaves, e.g. in spruce and ivy (Senser and Beck 1984). The meaning of this enlargement is still unknown.

The pigment content in blackberry leaves varies considerably throughout the year. The content of chlorophylls and carotenoids is especially high in winter. Previous investigations have shown that the concentration of the individual carotenoids changes throughout the season while their composition stays unchanged. The winter leaves contain more β-carotene, but less lutein than the summer leaves (Hloušek-Radojčić et al. 1985). Similar observations were also made by Huner et al. (1984) in hardened and nonhardened rye chloroplasts.

Several investigators have studied lipids in winter leaves and chloroplasts (O'Neil et al. 1981, Senser and Beck 1984, Havaux et al. 1984). All these authors have found changes either in the composition or in the quantity of lipids. Preliminary investigations of the leaves and isolated chloroplasts of blackberry show that there are significant differences in the composition of fatty acids between summer and winter leaves (Kunst 1986, personal communication).

Although winter blackberry leaves contain a high content of pigments, especially of chlorophylls, their photosynthetic activity is low. This is probably the consequence of changes in the macromolecular constituents of the thylakoids in hardened chloroplasts (Huner 1986). Griffits et al. (1984) stated that a large proportion of chlorophyll in winter rye was inactive in transferring energy to the photosynthetic reaction centers.

Winter blackberry leaves, like other cold hardened tissues, are able to endure repeated freezings and thawings and thus repeated dehydration and rehydration of their cells. During the freezing extracellular formation of ice crystalls causes dehydration of the cells (Pearce and Willison 1985). Genevès (1957) has shown that a detachment of the protoplasts from the cell walls appears in frozen leaves of *Iris*. This was only occasionally observed in frozen blackberry leaves. It seems therefore that the protoplasts either shrink together with their cell walls during the freezing of the tissue or that rehydration of the protoplasts occurs during the chemical fixation — necessary for electron microscopic investigation (Singh 1979).

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SAŽETAK

PROMJENE KLOROPLASTA U LISTOVIMA KUPINE TIJEKOM VEGETACIJSKE SEZONE

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Istražene su promjene u ultrastrukturi, sadržaju pigmenata i fotosintetskoj aktivnosti u kloroplastima listova kupine (Rubus fruticosus L. s. l.) tijekom proljeća, ljeta, jeseni i zime. Ultrastruktura mladih proljetnih listova pokazuje karakteristike mladog lisnog tkiva uz maksimalne vrijednosti fotosintetske aktivnosti. Tijekom ljeta i jeseni, usporedo s razvojem i starenjem tih listova, njihova ultrastruktura se mijenja. Kloroplasti ljetnih listova imaju dobro razvijen tilakoidni sustav i velika grana, a zrnca škroba redovito su prisutna. Sadržaj pigmenata tada dosiže maksimum, dok fotosintetska aktivnost postupno opada. Redukcija tilakoidnog sustava te povećanje broja i veličine plastoglobula obilježja su kloroplasta starih jesenjih listova. Njihova je fotosintetska aktivnost niska kao i sadržaj pigmenata i takvi listovi postupno propadaju. No, krajem ljeta i početkom jeseni izrastaju novi listovi koji se zadržavaju preko zime i propadaju tek u kasno proljeće. Kloroplasti zimskih listova imaju dobro razvijen tilakoidni sustav i malobrojna ali velika grana te obilnu stromu s dosta plastoglobula. Značajno je da su ti listovi tijekom jeseni i zime izloženi postupnom padu temperature i promjeni fotoperiode što uzrokuje njihovo prilagođavanje na niske temperature. Zimski listovi imaju velike kloroplaste i visok sadržaj pigmenata, no njihova fotosintetska aktivnost je relativno niska. Istražena je i ultrastruktura smrznutih zimskih listova na temperaturama ispod 0 °C (do — 12 °C). U stanicama tih listova redovito nalazimo više manjih vakuola i brojne vezikule, a gusta citoplazma potisnuta je između staničnih organela.

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