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Dedicated to Prof. dr. LJUDEVIT ILIJANIĆ on the occasion of his 70th birthday.

Structural and functional characteristics of overwintering blackberry leaves

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The structural and functional changes in overwintering blackberry leaves (*Rubus fruticosus* L.) were investigated. The changes in frozen and thawed leaves were studied by light and electron microscopy. In addition to conventional chemical fixation, the frozen leaves were also examined by microwave-enhanced chemical fixations, and by freeze-substitution methods.The winter leaves were multivacuolated and contained chloroplasts with large grana. By light and electron microscopy it was shown that, in leaves frozen between –4 °C and –10 °C, some intercellular spaces lying near the stomata were much enlarged. In the cells of these leaves the cytoplasm was condensed and the organelles aggregated. These characteristics were registered in frozen tissue after all the fixation methods applied.After thawing, the normal structures of the leaf cells soon reappeared, as did the respiration and photosynthetic activity of the leaves. At the end of the winter period (beginning of March) the leaves still had the characteristics of winter leaves, although their hardening seemed to decline. Signs of senescence (large plastoglobules in the chloroplasts) were observed in the overwintered leaves in spring, some time before shedding.

Key words: *Rubus fruticosus*, overwintering leaves, freezing, thawing, ultrastructure, pigments, photosynthesis

Introduction

Deciduous trees and shrubs usually shed their leaves in autumn, at the end of the warm season. This process begins with senescence of the leaves, and ends with their detachment from the twigs. However, in some deciduous woody plants, leaves that sprouted in late summer or early autumn can overwinter on the plants. During the winter period these leaves have to endure a repeated freezings and thawings, and therefore have to be adapted to survive subzero temperatures.

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This *cold hardening* or *cold acclimation* is a complex process still not completely understood (YOSHIDA 1999). It is known that, during hardening, complex alterations in the lipid content of the plasmalemma and of the endomembranes occur (UEMURA and STEPONKUS 1999). At the same time, there is also an increase in osmolarity, caused by an accumulation of intracellular solutes, especially of sugars, which act as cryoprotectants (RÜTTEN and SANTARIUS 1988, SAUTER et al. 1996). It is further known, that s.c. antifreeze proteins, which accumulate in the apoplast regions, are supposed to inhibit the growth of ice crystals (GRIFFITH et al. 1992). Recently, a direct evidence of the involvement of cold-regulated genes in increasing the tolerance of plant cells to freezing has been reported (UEMURA and STEPONKUS 1999).

The cold hardening process begins in autumn with the decrease of nocturnal temperatures, and reaches its maximum in mid-winter (RÜTTEN and SANTARIUS 1988). The freezing tolerance of the leaves differs from species to species. Conifer needles are known to survive subzero temperatures of about –40 °C or below, and spinach leaves only about –10 °C (SENSER and BECK 1984, BAUER et al. 1994). The leaves of blackberry belong to medium-hardy plants and are supposed to survive temperatures around –20 °C. A similar level of freezing tolerance has also been found for the leaves of some other perennial plants, such as strawberry, ivy and holly (O´NEILL et al. 1981, SENSER and BECK 1984, RÜTTEN and SANTARIUS 1988).

It is well known that, during moderate (not lethal) freezing, ice does not enter the cells. By low-temperature scanning electron microscopy it was shown that, in frozen wheat and rye leaves, ice crystals accumulated outside the cells, in the intercellular spaces, particularly in the substomatal cavities (PEARCE 1988, PEARCE and ASHWORTH 1992). Extracellular ice acts as a nucleation site for water vapour, which is driven out of the cells. The cells therefore desiccate, and as a consequence, their volume decreases (ANDREWS 1996). The quantity of ice, which is formed, depends on the freezing temperatures. In rye leaves frozen at –2.4 °C ice was not abundant, but at lower temperatures it filled most of the intercellular spaces. The mesophyll cells were then highly desiccated and collapsed (PEARCE and ASHWORTH 1992). It is supposed that a strong desiccation, connected with irreversible damage of the plasmalemma, is one of the main causes of cell death in frozen tissue (SINGH 1979, WEBB and STEPONKUS 1993).

Studies on the ultrastructure of cell organelles during freezing and thawing of the leaves are scarce (SINGH 1979, HARVEY and PIHAKASKI 1989). Our previous investigations have shown, that blackberry leaves are very suitable for this type of study as, shortly after thawing, they are able to restore completely their respiration and photosynthetic activity (MODRUŠAN and WRISCHER 1987). Here we report on the structural changes in winter blackberry leaves observed during freezing and thawing, i.e. during dehydratation and rehydratation of the cells. Reappearance of respiration and photosynthetic activity in leaves after thawing was also measured, and was used as a test for their viability.

During the relatively long-lasting conventional chemical fixation of frozen leaves for electron microscope study, some rehydratation of the aldehyde-fixed tissue may occur. Therefore, to be able to demonstrate the native structures in

frozen leaves as reliably as possible, we used additional fixation methods, which were intended to stabilize cell ultrastructures very quickly.

Materials and Methods

Blackberry leaves (*Rubus fruticosus* L.) were collected in their natural habitats from late October till the end of April. Winter leaves frozen outdoors (to temperatures that varied between –3 °C and –12 °C), as well as those frozen in the laboratory (by slow cooling, about 1 °C/h) were used. For structural studies, frozen leaves not covered with ice were selected.

The following fixation methods were used:

I. Conventional chemical fixation in 1% glutaraldehyde in 0.05M cacodylate buffer (pH 7.2) at 2 °C for 1 h, followed by rinsing in buffer and postfixation in 1% OsO_4 for 2 h. In the case of frozen winter leaves the temperature of the fixative was adjusted to outdoor temperatures. For fixation at temperatures lower than –5 °C, 5% of ethylene glycol (as an antifreeze agent) was added to the fixatives.

II. Microwave-enhanced fixation in 1% glutaraldehyde in cacodylate buffer (pH 7.2) for 30 seconds, followed by rinsing in buffer (5–10 min) and microwave-fixation in 1% OsO_4 for 30 seconds (HEUMANN 1992). Variation: microwave-enhanced simultaneous fixation with 1% glutaraldehyde plus 1% OsO_4 in cacodylate buffer for 30 seconds (WRISCHER et al. 1999).

III. Rapid freezing by plunging pieces of frozen leaves into liquid nitrogen, followed by freeze-substitution in 1% OsO_4-methanol at –80 °C for 7 days (PLATT and THOMSON 1996). Variation: Leaf pieces were directly plunged into 1% OsO_4-methanol at –80 °C and left there for 7 days.

The samples were embedded in Araldite, and thin sections, stained with uranyl acetate and lead citrate, were examined with a Zeiss EM 10 electron microscope. For light microscopy semithin sections of araldite-embedded tissue, stained with toluidine-blue were examined with the light microscope Zeiss Axiowert 35.

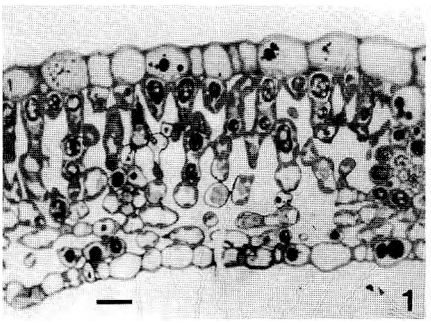
Pigments were extracted in 80% acetone and their concentrations calculated according to LICHTENTHALER (1987). Respiration and photosynthetic activity (production of oxygen) were measured with an oxygen electrode as described previously (WRISCHER et al. 1998).

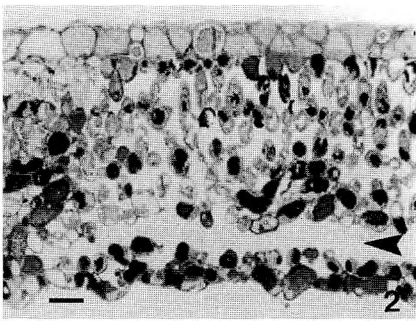
Results

Winter leaves examined before freezing

In early autumn the leaves that were to overwinter contained cells with large central vacuoles and peripherally arranged organelles. During late autumn and early winter, this arrangement changed. As observed by light microscopy, the winter leaves were thin with large intercellular spaces (Fig. 1). By electron microscopy it was seen that the cells, instead of a single large vacuole, contained several smaller ones, in addition to numerous vesicles, which are probably com-

ponents of the endoplasmic reticulum. In some vacuoles, there were large dark inclusions of unknown content. The cytoplasm was normally structured. The chloroplasts were large, with particularly well developed grana; grana with as many as 50° thylakoids were occasionally found. The chloroplast stroma was also abundant. Starch grains were occasionally present, and there were always some plastoglobules. The mitochondria and peroxisomes were normally developed, and dictyosomes were also occasionally found (Fig. 3).





Figs. 1–2. Semithin sections through winter leaves. Bars = 20 μm.

Fig. 1. Cross-section through winter leaf. Conventional chemical fixation.

Fig. 2. Cross-section through winter leaf frozen at –10 °C. Conventional chemical fixation + 5% ethylene glycol. Arrowhead = enlarged intercellular space.

Frozen winter leaves

For better interpretation, the frozen leaves were examined by several fixation procedures: by conventional chemical fixation, by microwave-enhanced fixation, and by the freezing and freeze-substitution methods. As shown by light microscopy on semithin sections, the structures of frozen leaves differed from those before freezing (Fig. 2). Some intercellular spaces situated near the stomata were much enlarged, while the mesophyll cells were displaced. Due to these large spaces, the thickness of the leaves increased by approximately 20%. At the same time, the cells collapsed somewhat, as compared to those of unfrozen leaves.

Figs. 3 – 6. Thin sections through overwintering leaves. ch = chloroplast, m = mitochondrion, n = nucleus, s = starch, v =vacuole, arrowhead = invaginations of the chloroplast envelope. Bars = 1 μm.

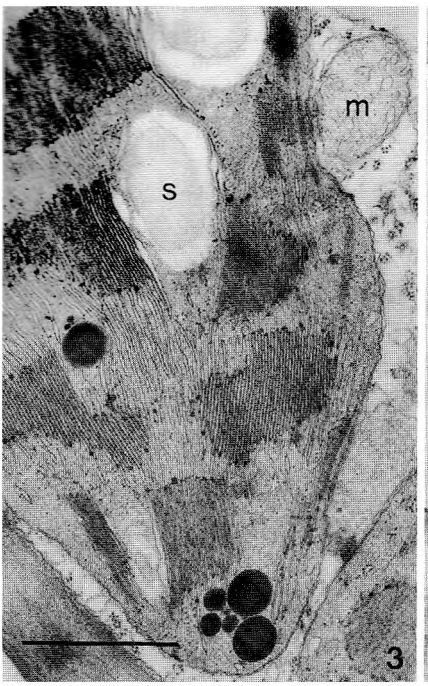
Fig. 3. Part of a cell from a winter leaf (beginning of December) with a large chloroplast. Conventional chemical fixation.

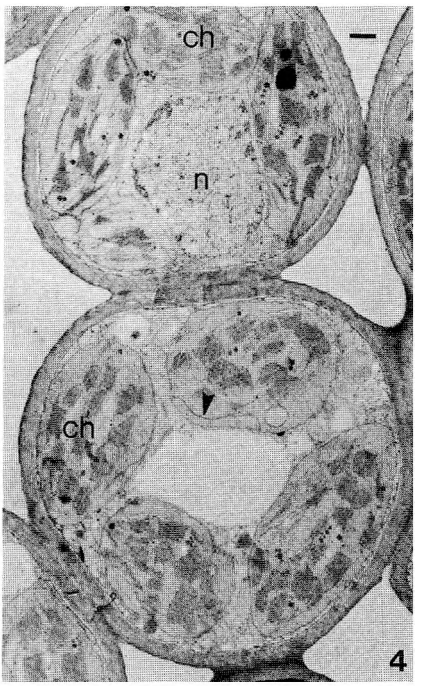
Fig. 4. Cells of a winter leaf frozen at –10 °C with tightly packed organelles. Conventional chemical fixation + 5% ethylene glycol.

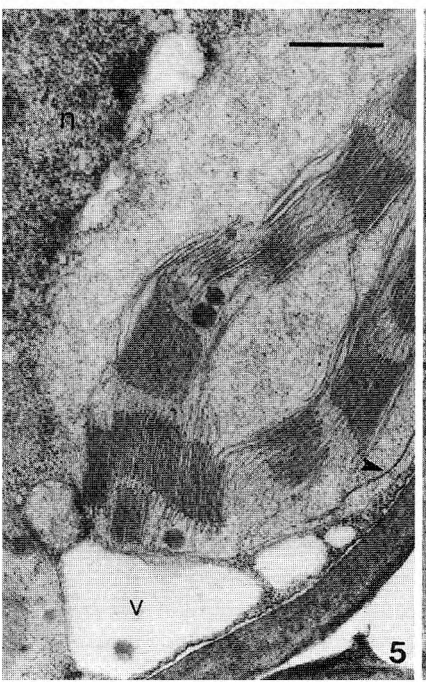
Fig. 5. Part of a cell from a winter leaf frozen at –4 °C with chloroplast and small vacuoles in the cytoplasm. Conventional chemical fixation.

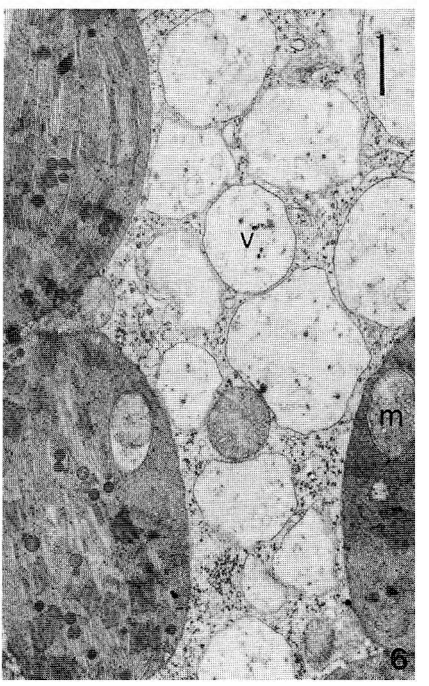
Fig. 6. Cell of a winter leaf frozen at –4 °C, and examined after one hour of thawing. There are numerous small vacuoles in the cytoplasm. Conventional chemical fixation.

Similar changes in frozen leaves were observed regardless of the fixation procedure applied.









The ultrastructure of frozen leaves, subjected to standard chemical fixation, is shown in Figs. 4 and 5. The cell organelles were aggregated, and pushed into one part of the cell, or they were crowded around the nucleus (Fig. 5). The cytoplasm was condensed, as observed by tightly packed ribosomes. In the cytoplasm there were several vacuoles, often with irregular outlines, as well as numerous small vesicles. The plasmalemma was attached to the cell wall, as in unfrozen leaves. The chloroplasts were well preserved, with an abundant stroma and large grana. In leaves frozen outdoors, the chloroplasts were starch-free. A characteristic found only in chloroplasts of frozen leaves were long, straight or convoluted, membranous invaginations of the inner membrane of the chloroplast envelopes. Mitochondria and peroxisomes were well recognizable and often appressed to the chloroplasts. Addition of the antifreeze agent, ethylene glycol, to the fixative (necessary for fixation at temperatures lower that –5 °C), did not visibly influence the preservation of cell ultrastructure (Fig. 4).

To verify the correctness of the ultrastructural image of frozen leaves obtained by conventional chemical fixation; additional fixation methods were used.

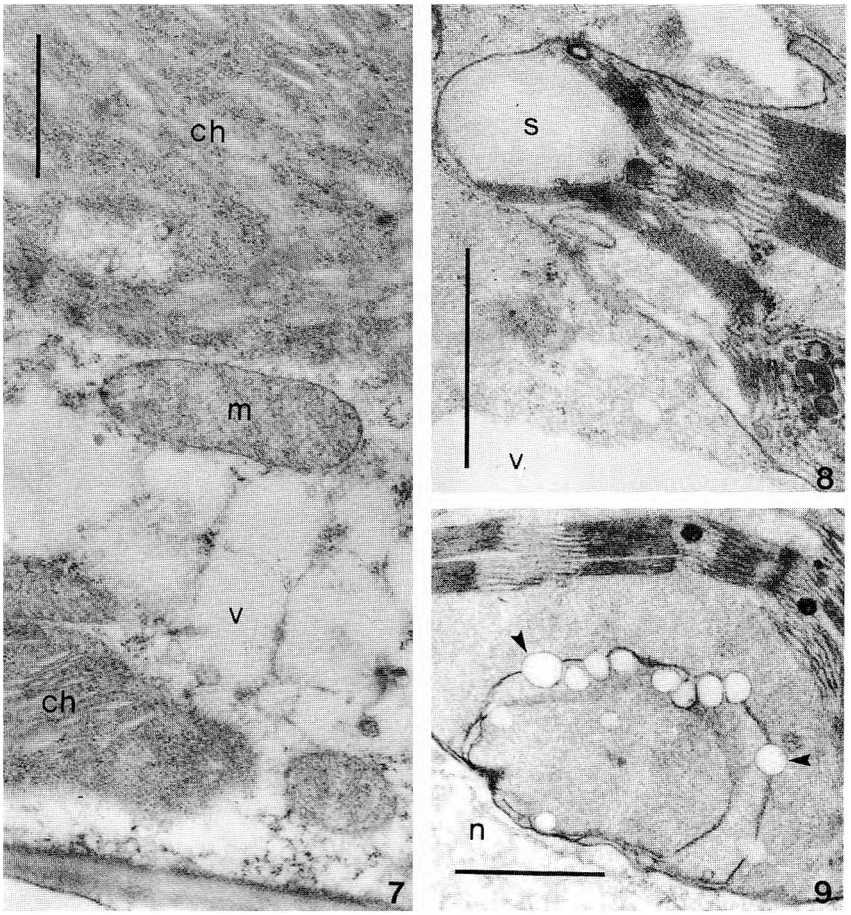
After microwave enhanced double fixation (separate treatments with glutaraldehyde- and OsO_4-fixatives) the picture of frozen leaf cells was similar to that found after conventional chemical fixation. The very rapid microwave fixation in a mixture of glutaraldehyde- and OsO_4-fixative, which lasted only 30 seconds, presented the cell ultrastructures well. In the chloroplasts only, the intrathylakoidal spaces were somewhat dilated. After this fixation all structures had a somewhat coarse appearance, which appears to be a consequence of the predominant effect of the OsO_4 in the fixative. Due to the very short fixation time and the slow penetration of this fixative into the tissue, only a peripheral layer of the sample could be used for analysis (Fig. 7).

After rapid freezing in liquid nitrogen, followed by freeze-substitution fixation, in mesophyll cells of the deeper – better preserved – regions of the sample, the cytoplasm was very dense, containing well discernible ribosomes. The chloroplasts were large, with darkly stained membranes, and with tightly appressed thylakoids in the grana regions. The membranous invaginations of the chloroplast envelopes were also detectable. On the contrary, the membranes of the vacuoles and small vesicles were only faintly stained (Fig. 8).

After freeze-substitution fixation (only in OsO_4-methanol) the cytoplasm and the nucleus of the leaf cells were less well preserved than the chloroplasts. Unusual were also some local dilatations of the chloroplast envelopes and their invaginations, indicating that, during fixation, some damage might occur (Fig. 9).

Winter leaves after thawing

After thawing, the large intercellular spaces observed in frozen leaves by light microscopy disappeared within a short time, and the leaves returned to the normal state of the winter leaf. After only one hour after thawing at room temperature, the ultrastructure of the leaf cells looked like that in leaves before freezing. The cells were multivacuolated, and in the cytoplasm the ribosomes were again loosely arranged. Mitochondria with well-developed tubules were frequently ob-



Figs. 7 – 9. Thin sections through frozen winter leaves. ch = chloroplast, m = mitochondrion, n = nucleus, s = starch, v = vacuoles. Bars = 1 μm.

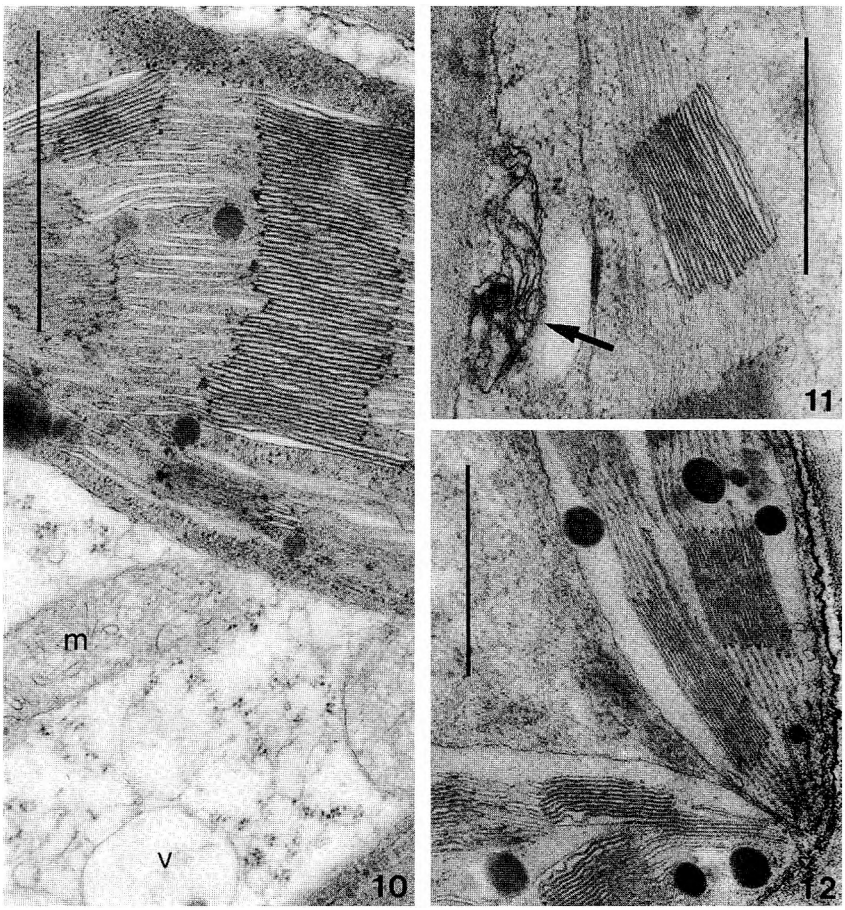
Fig. 7. Cell of a winter leaf frozen at -4 °C. Simultaneous microwave fixation.

Fig. 8. Cell of a winter leaf frozen at -5 °C. Part of a chloroplast with tightly packed grana thylakoids. Deep freezing + freeze-substitution fixation. (liquid nitrogen/OsO_4-methanol at -80 °C).

Fig. 9. Part of a chloroplast from a winter leaf cell frozen at -5 °C. Arrowheads = dilated invaginations of the chloroplast envelope. Freeze-substitution fixation in OsO_4-methanol at -80 °C.

served within chloroplast invaginations. The long membranous invaginations of the chloroplast envelope were not found in this material. Starch grains reappeared in the stroma (Fig. 6).

In late February and early March, the leaves still had some characteristics of winter leaves. The cells were multivacuolated, and the chloroplast grana were large (Fig. 10). In spite of these characteristics, freezing these leaves at –12 °C in the laboratory caused irreversible cell damage. After one hour of thawing, damage of the plasmalemma and of mitochondrial membranes was obvious. The



Figs. 10 – 12. Thin sections through overwintered leaves. m = mitochondrion, v = vacuole. Bars = 1 μm.

Fig. 10. Cell of an overwintered leaf (March). Chloroplast with large grana. Conventional chemical fixation.

Fig. 11. Cell of an overwintered leaf (March) frozen (in the laboratory) at –12 °C, and examined after one hour of thawing. Arrow = damaged endomembranes. Conventional chemical fixation.

Fig. 12. Part of a cell from an overwintered leaf (April). Chloroplasts with large plastoglobules. Conventional chemical fixation.

chloroplast stroma was empty and the thylakoids were disarranged (Fig. 11). Although the freezing was slow (1 °C/h), the question remains of whether it was comparable to that occurring in nature.

The overwintered leaves remained on the twigs until late spring, when new leaves were already sprouting. The multivacuolation of the cells gradually disappeared. Signs of senescence of these leaves were best detectable in the chloroplasts, in which plastoglobules became numerous and large (Fig. 12).

Content of pigments, respiration, and photosynthetic activity

The pigment content of overwintering leaves is shown in Tab. 1. The ratio of chlorophyll *a* to chlorophyll *b* was 1.75, and that of total chlorophylls to total carotenoids 4.49.

Photosynthetic activity (production of oxygen) in overwintering leaves was high, but its efficiency, with respect to the chlorophyll level, was relatively low (Tab. 1). The respiration of the winter leaves is shown in Tab. 1 as well.

When frozen leaves were left at room temperature, respiration and photosynthetic activity could soon be detected. The speed of reappearance depended on the temperature to which the leaf had previously been frozen. In leaves frozen to –4 °C, and examined one hour after the start of thawing, both respiration and photosynthesis were already high (45.20 μmol O_2/h/ mg fr. wt., and 126 μmol O_2/h/ mg fr. wt.). Several hours later the values found in unfrozen winter leaves (Tab. 1) were reached. At the end of the winter period, with outdoor temperatures above freezing point, leaves did not endure strong freezing (to –12 °C) in the laboratory. In these leaves, after one hour of thawing, both respiration and photosynthesis were very low, and three hours later they stopped completely.

Tab. 1. Total chlorophylls, total carotenoids, photosynthetic activity, respiration, and photosynthetic efficiency of winter blackberry leaves.

Total chlorophylls (mg/g fr. wt.)	Total carotenoids (mg/g fr. wt.)	Photosynthetic activity (μmol O_2/g fr. wt./h)	Respiration (μmol O_2/g fr. wt./h)	Photosynthetic efficiency (μmol O_2/mg chl./h)
2.23±0.07	0.52±0.56	185.27±1.76	52.41±3.79	83.07±0.79

Discussion

Winter leaves of the blackberry exhibit some peculiarities, which distinguish them from summer leaves. Their chloroplasts are larger than those of summer leaves (MODRUŠAN and WRISCHER 1987). Nevertheless, the photosynthetic activity of winter leaves, expressed with respect to chlorophyll content, is low. The large grana and the high pigment content in these leaves may be a consequence of the low light conditions existing in late autumn and in winter.

Another peculiarity of winter blackberry leaves is their type of vacuolization. An increase in the compartmentalization of the cytoplasm of winter leaves was previously described for strawberry (O´NEILL et al. 1981) and rye (HUNER et al. 1984). In winter, in cells from the woody tissue of poplar, SAUTER et al. (1996) proved a correlation between the presence of numerous small vacuoles and vesicles (components of the endoplasmic reticulum), and the high sugar content of these cells. Decrease in hardening was again paralleled by a decrease in both sugar content and the population of vesicles. The change in compartmentalization of the cytoplasm seems therefore to be connected with the biochemical processes during hardening. In addition to the accumulation of sugars, an increase in lipid levels was reported (O´NEILL et al. 1981, SENSER and BECK 1984).

SAUTER et al. (1996) supposed that changes in the vacuolization of the hardened winter cells indicated activity of an intrinsic dehydration system, which acted during freezing by withdrawal of water from the protoplasts into the extracellular space.

It is known that freezing of the leaves gives rise to the accumulation of ice in the intercellular spaces (PEARCE and ASHWORTH 1992). On semithin sections of araldite-embedded frozen blackberry leaves we noticed the enlargement of some intercellular spaces. These enlarged spaces were detected in frozen leaves prepared by different fixation techniques. We therefore suppose that they reflect the real structure of frozen tissue. They probably contained ice before fixation, which was solubilized during preparation. Connected with this phenomenon were a slight dehydratation and collapse of the cells of frozen leaves, observed with both the light and the electron microscope. With the electron microscope, aggregation of the cell organelles and condensation of the cytoplasm were well visible. HARVEY and PIHAKASKI (1990) supposed that the vesicles found in frozen rye leaves were additional membrane material needed for the enlargement of the plasmalemma during defreezing, i.e. rehydratation of the cells. In our material (blackberry leaves) small vesicles were present in the cytoplasm of both frozen and unfrozen leaves, although their number and dimensions might vary. The significance of the enlargement of the inner membrane of the chloroplast envelopes, in the form of long invaginations into the stroma, found in frozen leaves is for the moment unknown. It has been suggested that similar structures in the chloroplasts of frozen winter leaves of spruce and ivy represent an adaptation to low temperatures (SENSER and BECK 1984).

A comparison of pictures obtained with different fixation techniques revealed slight differences in the presentation of the ultrastructures of frozen leaves. The quick simultaneous microwave-enhanced fixation, as well as deep-freezing, followed by freeze-substitution fixation, present the ultrastructures of frozen leaves well, but only in well preserved tissue portions. Less well-preserved tissue was obtained with the freeze-substitution fixation in OsO_4-methanol at –80 °C. The cytoplasm and the chloroplast envelopes particularly indicated possible fixation damages. Finally, the results obtained with conventional chemical fixation indicated that this method gave reliable results and was thus suitable for the analysis of frozen tissue. As expected, a slight loosening of some structures, particularly of the cytoplasm, during fixation and rinsing in aqueous medium could not be completely excluded.

At the end of the winter period (late February and early March) blackberry leaves did not endure freezing to low temperatures. It is known that, in winter, cold hardening decreases during a transient rise of the temperature above the freezing point (SAUTER et al. 1996). On the other hand, in our material, we cannot completely exclude the possibility that the freezing performed in the laboratory was too fast and could thus damage the tissue.

In spring univacuolation of the overwintered leaf cells reappeared. At the same time, leaf senescence, which had stalled during the cold winter months, started again. In chloroplasts this is clearly reflected by a decrease in size (MODRUŠAN and WRISCHER 1987). At the same time, the number and dimensions

of plastoglobules increase, which is a characteristic sign of chloroplast senescence (LJUBEŠIĆ 1976).

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