Acta Bot. Croat. 62 (1), 1–10, 2003

CODEN: ABCRA 25
ISSN 0365–0588

Chloroplast structure and function in wild-type and *aurea*-type leaves of the Japanese spindle-tree over their life span

NIKOLA LJUBEŠIĆ*, MERCEDES WRISCHER, TATJANA PREBEG, ZVONIMIR DEVIDÉ

Ruđer Bošković Institute, Department of Molecular Genetics, PO Box 180, HR-10002 Zagreb, Croatia

The evergreen leaves of the Japanese spindle-tree (*Euonymus japonicus* Thunb. 'Aureomarginatus') are adapted to endure repeated freezing and thawing during the winter. We investigated the physiological and morphological characteristics of the chloroplasts in young, overwintering, and overwintered wild-type and *aurea*-type leaves. When developing in the shade, both leaf types during their life span maintained a well-developed thylakoid system in their chloroplasts. The photosynthesis rates were also similar. When growing in intense sunlight, the *aurea* variety contained chloroplasts with a reduced thylakoid system, but photosynthesis was less reduced. In spring, overwintered leaves of both varieties clearly showed ultrastructural and physiological characteristics of senescence. The senescence in *aurea* leaves exposed to full sunlight was more pronounced.

Key words: *Euonymus japonicus,* wild-type plant, *aurea*-variety, overwintered leaves, chloroplast, ultrastructure, photosynthesis

Introduction

The life span of the leaves of the evergreen Japanese spindle-tree (*Euonymus japonicus* Thunb.) normally extends through two vegetative periods. The leaves sprout in spring, mature in summer and begin to senesce in autumn. They overwinter on the shrub, however, and are not shed until the next generation of leaves appears, in spring or early summer (LISICA 1998).

The cultivar 'Aureomarginatus' bears, in addition to wild-type leaves, also those with *aurea* characteristics, which are very sensitive to intense illumination (WRISCHER et al. 1986). They become green only in the shade, but turn yellow when exposed to full sunlight. After prolonged exposure to strong illumination, they bleach and detach from the plants.

To be able to endure winter subzero temperatures the leaves of evergreen plants have to be »hardened« during autumn by special biochemical processes (YOSHIDA 1999). This also applies to the leaves of the Japanese spindle-tree, both of the wild and of the *aurea* type.

* Corresponding author: Fax: +385-1-4561-177, e-mail: ljubesic@rudjer.irb.hr

The developmental pattern of the chloroplasts of this species, during their whole life span, was not well known. The prolonged period of leaf senescence throughout autumn, winter and spring was of particular interest. Here we describe the structural and functional changes in leaves and chloroplasts of both wild-type and *aurea*-type plants exposed to different light intensities, during two vegetative periods.

Materials and Methods

The leaves of the Aureomarginatus cultivar of the Japanese spindle-tree usually have a dark green centre and intensely yellow margins. Occasionally, however, branches with completely green (wild-type) or yellow (*aurea*-type) leaves appear. Taking cuttings from such branches of a single shrub, we raised plants with only wild-type, or only *aurea*-type, leaves. All our experiments were performed with leaves from these genetically uniform clones.

Wild-type and *aurea*-type plants of the Japanese spindle-tree (*Euonymus japonicus* Thunb. 'Aureomarginatus') were grown in a private garden in Zagreb. Leaf structure and photosynthesis were studied: in spring (April, May), in summer (July, August), in autumn (October, November), in winter (January, February), as well as in the spring (April, May) of the next growth season. The examinations were repeated in two consecutive blocks of two growth periods.

Small pieces of leaf tissue were fixed in 1% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) at 2 °C for 1 h. After rinsing in buffer, they were postfixed in 1% OsO_4. Cytochemical localisation of photosynthetic activity (photooxidation of DAB) was performed according to WRISCHER (1989). After dehydration, the samples were embedded in araldite or in Spurr's medium. The sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 10A electron microscope. For light microscopy, semithin sections of embedded tissue were stained with toluidine blue and examined with the light microscope Zeiss Axiowert 35.

Pigments were extracted with 80% acetone and their concentrations were calculated according to LICHTENTHALER (1987). Photosynthetic activity (production of oxygen) of slices of leaves was measured with a Clark-type oxygen electrode (Hansatech Ltd., London). The reaction mixture contained 0.1mol L^{-1} K-Na-phosphate buffer (pH 7.2) and 0.01 mol L^{-1} Na-bicarbonate. The samples were illuminated with a halogen lamp at saturating illumination at 25 °C (WRISCHER et al. 1998).

Results

Wild-type leaves

The wild-type leaves, which sprouted in spring (April), were light green. Those growing inside the shrub, and therefore shaded by other leaves, were somewhat more intensely green than those growing at the periphery of the shrub, and thus exposed to full sunlight. During summer and autumn, the leaves increased in size and became dark green. They then overwintered on the shrubs and endured several freezing and thawing periods. Only in the next spring and summer, just before their shedding, did they start to yellow.

The chlorophyll content increased constantly in the leaves till the next spring. At the same time the amount of carotenoids changed only slightly (Tab. 1). The highest photosynthetic activity was measured in winter leaves, but dropped in old overwintered leaves, in spring (Tab. 1). There were slight differences between sun and shade leaves.

Tab. 1. Content of total chlorophylls (**chl *a+b***), total carotenoids (**car**) and **photosynthetic activity** expressed as mol O_2 g^{-1} FW h^{-1} (A) and as mol O_2 mg^{-1} chlorophylls h^{-1} (B) in leaves of *Euonymus japonicus* exposed to different light intensities. Measurements were performed throughout two growth periods (mean value standard deviation).

		wild-type leaves				*aurea*-type leaves			
		chl *a+b* mg g^{-1} FW	**car** mg g^{-1} FW	**photosynthetic activity** A	B	**chl *a+b*** mg g^{-1} FW	**car** mg g^{-1} FW	**photosynthetic activity** A	B
late spring	full sunlight	1.001	0.223	78.40	78.00	0.021	0.174	–	–
		0.213	0.019	8.22	8.12	0.003	0.012		
	shade	1.076	0.265	85.47	79.46	0.341	0.148	114.61	336.13
		0.222	0.025	7.93	6.38	0.092	0.011	16.33	26.47
winter	full sunlight	0.971	0.261	109.43	112.74	0.105	0.121	38.67	367.64
		0.242	0.016	15.90	14,76	0.033	0.009	9.89	28.33
	shade	1.029	0.234	117.86	114.46	0.627	0.163	93.11	148.56
		0.203	0.021	22.43	16.64	0.080	0.010	18.86	22.87
second spring	full sunlight	1.196	0.242	63.1	52.78	0.052	0.117	–	–
		0.301	0.022	10.89	8.72	0.005	0.013		
	shade	1.435	0.267	64.24	44.75	1.043	0.129	91.38	87.65
		0.321	0.019	8.82	6.38	0.307	0.010	14.88	12.93

Light microscopy revealed that leaves growing in the shade were thinner (0.35 mm) than those growing in full sunlight (0.45 mm) (Figs. 1 a, b). Their thickness did not change markedly during the rest of their life span.

In **spring** (April), young leaves growing in the shade contained chloroplasts with grana of 3–15 thylakoids. The stroma contained a large number of ribosomes, starch grains, and just a few small plastoglobules. In the plastids of sun-exposed leaves, the grana contained a somewhat smaller number of thylakoids and the starch grains were smaller. During **summer and autumn**, in dark green leaves growing in the shade, the chloroplasts were large, with large grana stacks. Plastoglobules increased in size, but starch grains were not visible in autumn leaves. In **winter** (January and February) the leaves passed through repeated freezings and thawings. When examined in winter, while temperatures were slightly above zero, the chloroplasts contained very large grana and abundant stroma (Fig. 4). In addition, long invaginations of the inner membrane of the chloroplast envelope protruded into the stroma (Fig. 4, arrowhead). In the cells of winter leaves, there were additional characteristic changes, such as shrinking of the cytoplasm and the appearance of numerous small vacuoles, instead of a single central one (WRISCHER et al. 2000). In **spring** (April), the cytoplasm reverted to its normal (i. e. summer and autumn) appearance. At this time, the

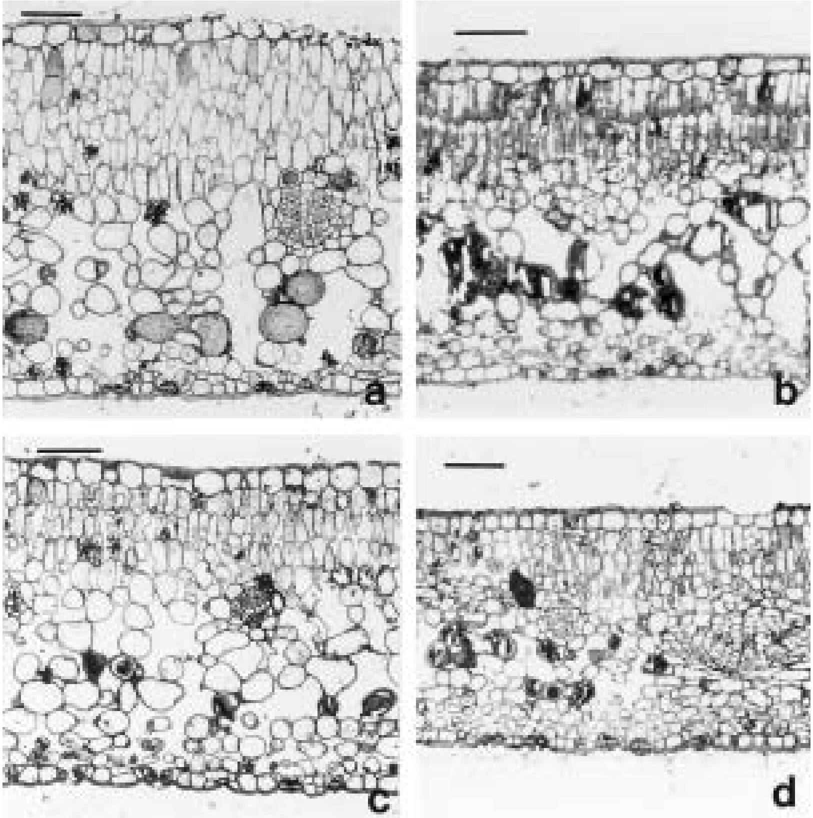


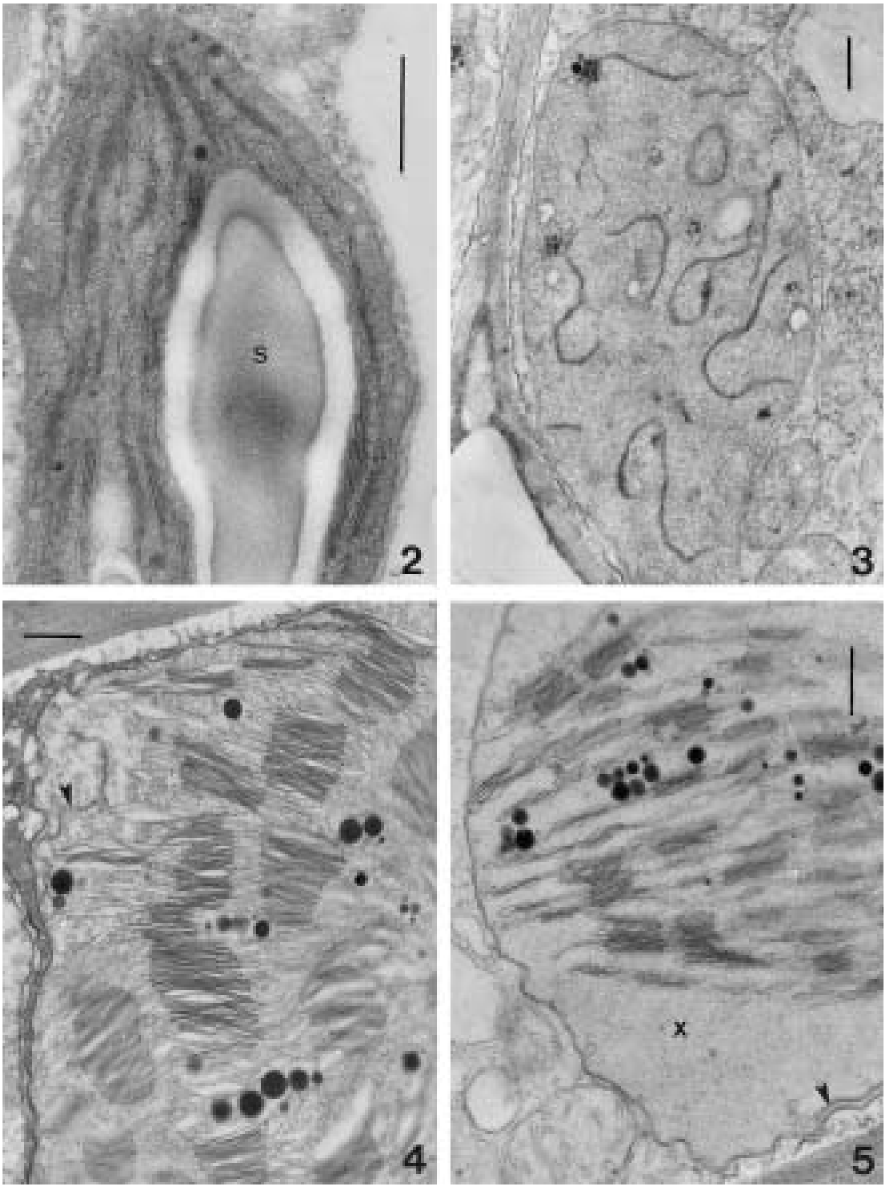
Fig. 1. Semithin transversal sections through leaves of *Euonymus japonicus*. Bar = 100 m. a – Wild-type leaf growing in the shade. b – Wild-type leaf growing in full sunlight. c – *aurea*--type leaf growing in the shade. d – *aurea*-type leaf growing in full sunlight.

chloroplasts of the overwintered, still green, leaves that were growing in the shade had large grana, which were irregularly arranged in the stroma, and there were groups of large plastoglobules (Fig. 8). In the chloroplasts of sun-exposed leaves, plastoglobules were particularly abundant (Fig. 9). The subsequent process of senescence followed a similar pattern in leaves from the shade and from full sunlight: complete disintegration of the thylakoid system and formation of numerous plastoglobules.

Aurea-type leaves

In **spring** (April), only those young leaves that developed in the shade became light green, while those growing at the periphery of the shrub, and thus exposed to full sunlight, were more or less yellow. After prolonged insolation the yellow leaves turned white and, after about three months, they detached from the branches. However, the green leaves and the majority of the yellow-green leaves overwintered and were not shed until late spring.

Chlorophyll levels in shade-grown leaves were significantly higher than in leaves from full sunlight (in spring 16 times, in winter 6 times and in overwintered leaves, in the next



Figs. 2–5. Thin sections through leaves of *Euonymus japonicus*. s – starch, arrowheads – invagination of the inner membrane of the envelope, x – enlarged stroma. Bar = 0.5 m.

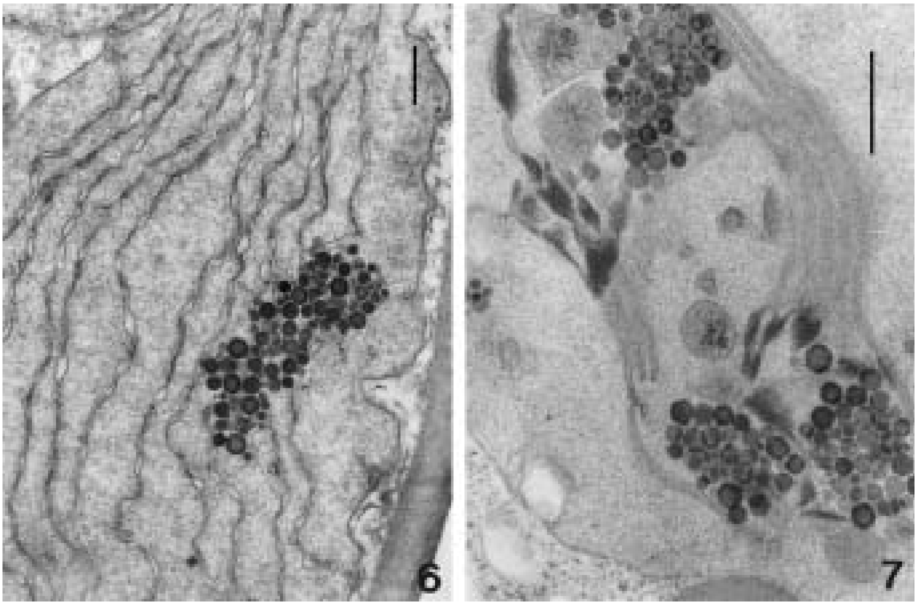
Fig. 2. Chloroplast from a young *aurea* leaf growing in the shade, sampled in spring. There are small grana and starch grains in the stroma.

Fig. 3. Plastid from a young *aurea*-type leaf growing in full sunlight, sampled in spring. Only single thylakoids developed.

Fig. 4. Chloroplast from a wild-type winter leaf growing in the shade, showing large grana and plastoglobules in the stroma.

Fig. 5. Chloroplast from an *aurea*-type winter leaf growing in the shade, with grana and numerous plastoglobules.

spring, 20 times). The concentrations of chlorophylls *a* and *b* peaked in winter (Tab. 1). Carotenoid levels did not vary markedly and were usually higher (10–20%) in shade-leaves. Only in young spring leaves did exposure to full sunlight lead to a slight increase (15% higher carotenoid concentrations than in shade leaves) (Tab. 1).

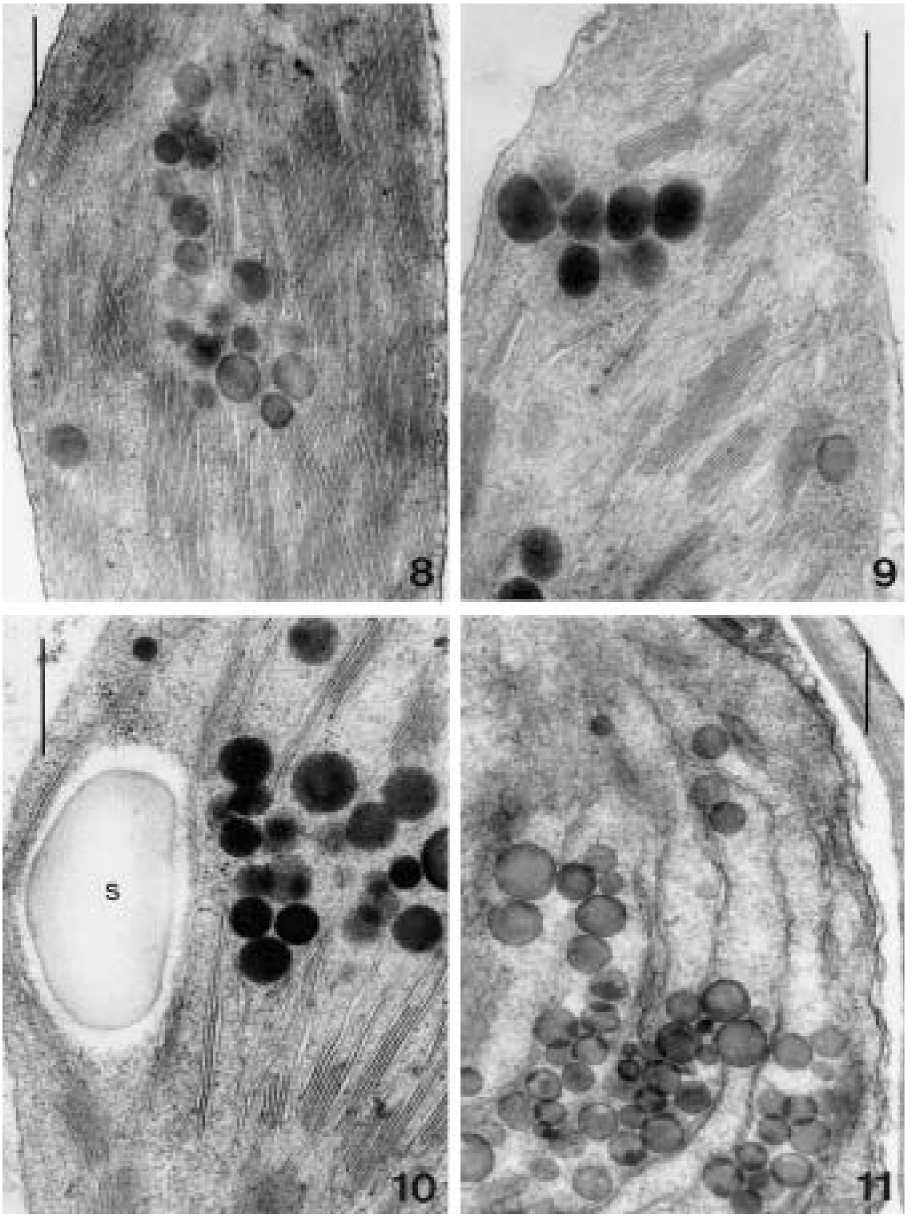


Figs. 6–7. Thin sections through leaves of *Euonymus japonicus*. Bar = 0.5 m.
Fig. 6. Plastid from a yellow *aurea*-type winter leaf growing in full sunlight, containing single thylakoids and accumulations of plastoglobules.
Fig. 7. Plastid from a yellow (bleached) *aurea*-type winter leaf growing in full sunlight, with single thylakoids and accumulations of plastoglobules.

The photosynthetic activity of sun-exposed spring leaves and overwintered leaves was extremely low (often unmeasurable), while there was definite photosynthesis (and an increased chlorophyll level) in winter (Tab. 1). In contrast, light-green shaded leaves had high photosynthetic activity during the whole period of observation. Maximal values were reached in late spring and summer. During winter, the photosynthetic rate remained relatively high (only 10% lower than in summer) and began to decrease in overwintered leaves, in the next spring (Tab. 1).

The average thickness of *aurea*-leaves exposed to full sunlight was about 0.4 mm, as compared to 0.3 mm for leaves developing in the shade, and thus somewhat less than for wild-type leaves (Figs. 1c, d). This difference was particular distinct for the leaves developing in the shade.

The ultrastructure of chloroplasts from **young spring** leaves depended on their position in the shrub. In leaves growing in the shade, there were small grana of 2 to 5 thylakoids, and the stroma contained some starch grains and small plastoglobules (Fig. 2). On the other hand, in plastids of sun-exposed, yellow leaves, the thylakoid system consisted of single



Figs. 8–11. Thin sections through leaves of *Euonymus japonicus*. s – starch. Bar = 0.5 m.

Fig. 8. Chloroplast from an overwintered wild-type leaf growing in the shade. There are large grana and numerous plastoglobules.

Fig. 9. Chloroplast from an overwintered wild-type leaf growing in full sunlight with grana and large plastoglobules in the stroma.

Fig. 10. Chloroplast from an overwintered *aurea*-type leaf growing in the shade, containing grana, starch and numerous plastoglobules.

Fig. 11. Plastid from an overwintered *aurea*-type leaf growing in full sunlight, with single thylakoids and numerous large plastoglobules.

thylakoids or pairs of tylakoids (Fig. 3). Starch was usually missing and the few plastoglobules were small. When the yellow leaves were shaded, e.g. when they were overgrown by other leaves, small grana appeared in their chloroplasts after some time. On the other hand, further bleaching of the yellow leaves caused drastic changes in the ultrastructure of the plastids. In an empty stroma, there were thylakoids arranged into grana-like structures. These membranes were diaminobenzidine-negative, i.e. photosynthetically not active (data not shown).

Characteristically, the plastids of **autumn leaves** (November) contained large groups of plastoglobules in the stroma. In green leaves from the shade, the chloroplasts had a normally developed thylakoid system. In yellow, sun-exposed, leaves the plastids contained only single thylakoids (WRISCHER et al. 1976).

During **winter,** the chloroplasts of both green (shade) and yellow-green leaves (full sunlight) had well developed grana, but, in a similar fashion as in wild-type chloroplasts, there was an increase in stromal material, and there were numerous invaginations of the inner membrane of the envelope (Fig. 5). In yellow leaves, growing in strong light, the thylakoid system was much reduced, and the number of plastoglobules increased considerably (Figs. 6, 7). The majority of these leaves abscised during winter or early spring.

In **spring,** the large chloroplasts of overwintered green leaves (from the shade) contained large starch grains. In these chloroplasts, the grana consisted of several thylakoids and there were some plastoglobules scattered in the stroma (Fig. 10). On the other hand, plastids from sun-exposed overwintered *aurea*-leaves contained only a few single thylakoids and numerous large plastoglobules, accumulated in groups (Fig. 11). The process of disintegration of the thylakoid system, and formation of gerontoplasts, was similar under both light conditions.

Discussion

The leaves of the Japanese spindle-tree, both of the wild- and of the *aurea*-type, are able to endure winter temperatures as low as *ca.* –20 °C. They thus belong to the group of medium-hardy plants, such as ivy (SENSER and BECK 1984), holly (RÜTTEN and SANTARIUS 1988), or blackberry (MODRUŠAN and WRISCHER 1987). There were no conspicuous differences in the hardening ability between wild-type and *aurea*-type leaves.

The developmental pattern of *aurea* leaves, when growing in the shade, was similar to that of wild-type leaves. However, their photosynthetic activity, when expressed with respect to chlorophyll concentration, was always very high. When growing in strong sunlight, *aurea* leaves soon started to bleach, and were detached from the twigs within maximally three months. The *aurea*-type leaves of *Euonymus japonicus* thus react in a way similar to that of other very sensitive *aurea* leaves, like those of *Fraxinus excelsior* (WRISCHER et al. 1975).

Both the wild-type, and the *aurea*-type leaves (those growing in the shade) start to senesce in autumn. This is most obviously evidenced by an increase of the number of plastoglobules in the stroma. Their appearance is always a sign of chloroplast senescence (LJUBEŠIĆ 1968, LJUBEŠIĆ 1976). Due to low temperatures the process of senescence is delayed during the winter months, and continues again when temperatures start to increase in spring.

In spite of first visible signs of senescence, the *in vitro* rate of photosynthesis in wild-type winter leaves exceeded that of young spring and summer leaves. It is a puzzle so far why leaves (growing in the shade or exposed to full sunlight) that were exposed to subzero temperatures for prolonged periods of time should show such high photosynthetic rates. A similar effect was described for the winter leaves of the blackberry (MODRUŠAN and WRISCHER 1987, WRISCHER et al. 2000). In *aurea*-type leaves, this phenomenon was most clearly visible when photosynthetic activity was expressed with respect to the concentration of chlorophylls. Photosynthetic activity in relation to fresh weight showed this phenomenon in a less pronounced fashion.

Acknowledgement

The authors would like to thank Dr. Volker Magnus for the critical reading of the manuscript.

References

LICHTENTHALER, H. K., 1987: Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Meth. Enzymol. 148, 350–382.

LISICA, O., 1998: Strukturne i funkcionalne promjene u listovima kurike (*Euonymus japonicus* Thunb. 'Aureomarginatus') tijekom dviju vegetacijskih sezona. / Structural and functional changes in spindle tree (*Euonymus japonicus* Thunb. 'Aureomarginatus') during two vegetation seasons. B. Sc. Thesis, University of Zagreb.

LJUBEŠIĆ, N., 1968: Feinbau der Chloroplasten während der Vergilbung und Wiederergrünung der Blätter. Protoplasma 66, 369–379.

LJUBEŠIĆ, N., 1976: Phytoferritin in plastids of blackberry leaves. Acta Bot. Croat. 35, 51–55.

MODRUŠAN, Z, WRISCHER, M., 1987: Seasonal changes in chloroplasts of blackberry leaves. Acta Bot. Croat. 46, 23–31.

RÜTTEN, D. SANTARIUS, K. A., 1988: Cold acclimation of *Ilex aquifolium* under natural conditions with special regard to the photosynthetic apparatus. Physiol. Plant. 72, 807–815.

SENSER, M., BECK, E., 1984: Correlation of chloroplast ultrastructure and membrane lipid composition to the different degrees of frost resistance achieved in leaves of spinach, ivy and spruce. J. Plant Physiol. 117, 41–55.

WRISCHER, M., LJUBEŠIĆ, N., DEVIDÉ, Z., 1975: Ultrastructural studies of plastids in leaves of *Fraxinus excelsior* L. var. aurea (Willd.) J. Microscopie Biol. Cell. 23, 105–112

WRISCHER, M., LJUBEŠIĆ, N., DEVIDÉ, Z., 1975: Transformations of plastids in the leaves of *Acer negundo* L. var. *odessanum*. (H. Rothe). J. Cell Sci. 18, 509–518.

WRISCHER, M., HLOUŠEK-RADOJČIĆ, A., KUNST, LJ., LJUBEŠIĆ, N., 1986: Differentiation of chloroplasts in leaves of aurea plants. In: AKOYUNOGLOU, G., SENGER, H. (eds.), Regulation of chloroplast differentiation, Plant Biology Series., 685–690. A. R. Liss Inc. Publ., New York.

WRISCHER, M., 1989: Ultrastructural localisation of photosynthetic activity in thylakoids during chloroplast development in maize. Planta 177, 18–23.

WRISCHER, M., LJUBEŠIĆ, N., SALOPEK, B., 1998: The role of carotenoids in the structural and functional stability of thylakoids in plastids of dark-grown spruce seedlings. J. Plant Physiol. 153, 46–52.

WRISCHER, M., LJUBEŠIĆ, N., MAGNUS, V., DEVIDÉ, Z., 2000: Structural and functional characteristics of overwintering blackberry leaves. Acta Bot. Croat. 59, 5–16.

YOSHIDA, S., 1999: A prefatory note on responses of plants to low temperature-stress. J. Plant Res. 112. 223–224.