

Dedicated to Prof. dr. MERCEDES WRISCHER
on the occasion of her 70th birthday.

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CHROMOPLASTS IN THE SEPALS OF *PHYSALIS ALKEKENGII*: THE EFFECT OF NORFLURAZON ON CHROMOPLAST DIFFERENTIATION

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The differentiation of chromoplasts in the sepals of *Physalis alkekengi* was studied by light- and electron-microscopy and pigment analyses. The development of the chromoplasts from the chloroplasts started about four weeks after anthesis and, within the next two weeks resulted in the formation of small electron-transparent crystalloids connected to plastoglobules. Treatment of the sepals with norflurazon, which inhibits β -carotene accumulation, prevented the formation of the small crystalloids. Only numerous plastoglobules filled the chromoplasts. The results are discussed with regard to the already known action of some bleaching herbicides.

Key words: *Physalis alkekengi* L., sepals, chromoplast differentiation, carotenoid crystalloid, norflurazon, ultrastructure

Introduction

Chromoplasts are defined as photosynthetically inactive organelles which contain large amounts of carotenoid pigments. They often give rise to intense yellow, orange or red colors in the petals of various plant species. However, the

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color of the sepals is usually green. Only rarely do they change to non-green colors, e.g. in *Impatiens* flowers (WRISCHER et al. 1999). The petals of *Physalis alkekengi* are white and, after anthesis, the green sepals of the calyx rapidly grow and form a large bladder around the fruit. As the fruit matures, the calyx becomes intensely red colored. Early observations (LICHTENTHALER 1969) show the formation of plastoglobules in the chromoplasts of the mature fruit and sepals of *Physalis alkekengi*. However, the investigations of SIMPSON et al. (1978) and SELAKOVIĆ et al. (1996) indicated the differentiation of chromoplasts of the crystalline type. Our findings have shown that the mature fruit and the whole calyx contain characteristic crystalline chromoplasts. In the present report we describe the structural changes and carotenoid composition during chromoplast formation, and the effect of the bleaching herbicide, norflurazon, on these processes.

Material and Methods

Physalis alkekengi L. was grown outdoors in the Botanical Garden of the University of Zagreb. All experiments were performed in July, August and September, in two consecutive years. For our observations we used the sepals and fruits of control and norflurazon-treated plants at different stages of their development. Most studies on plastid development were carried out in the mesophyll cells of sepals.

An aqueous solution of the herbicide, norflurazon (SAN 9789; 4-chloro-5-(methylamino)-2-(α,α,α -trifluoro-m-tolyl)-3-(2H)-pyridazinone), at a (sublethal) concentration of 2×10^{-5} M, was injected into the lumen of the bladder formed by the sepals. The tips of the sepals were tied up with thread and lifted up, so that the fruit was in a horizontal position. In that way, the injected solution would stay in the bladder during the whole experiment (Fig. 1). About one-quarter of the volume of the bladder was filled with the herbicide solution.



Fig. 1. The position of the sepal bladder of *Physalis alkekengi* during norflurazon treatment.

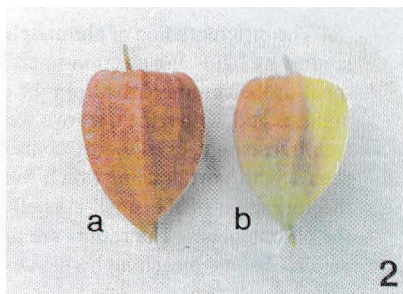


Fig. 2. Control (a) and norflurazon-treated (b) mature sepal-bladder of *Physalis alkekengi*.

Light microscopy was performed using Zeiss Axiovert 35 and Zeiss Axiolab microscopes.

For electron microscopy, pieces of control and norflurazon-treated sepal and fruit tissues were fixed in 1 % glutaraldehyde in sodium cacodylate buffer (pH 7.2) and postfixed in 1 % OsO_4 . The dehydrated tissue was embedded in araldite and ultrathin sections were prepared using an RMC, MT 6000-XL ultramicrotome

and stained with uranyl acetate and lead citrate. Sections of the mesophyll of the sepals and the subepidermal layer of fruit were examined in a Zeiss EM 10A electron microscope.

For pigment extraction the samples were cut into small pieces, mixed with a small amount of BaCO₃ and quartz sand, and ground in 100 % acetone in a mortar. For quantitative measurements the obtained carotenoids were separated by thin-layer chromatography on silica-gel G plates with a mixture of petrol ether (40–70 °C) and acetone (70:30). The bands that contained the carotenoids were eluted with acetone. The absorbance for carotenoids at 453.5 nm was measured with a Specol 10 spectrophotometer (Zeiss, Jena).

Results

Our observations have shown that there are no differences in plastid ultrastructures in fruit and sepals. As plastid differentiation could be more conveniently studied in the sepals than in the fruit, the investigations presented in this article were carried out on sepals.

Macroscopic observations

Control. The young immature fruit and sepals were intensely green. During ripening, their green color turned into yellow, then orange, and finally fully developed sepals and fruit attained an intensely red color (Fig. 2, a). This process was completed within about four weeks.

Norflurazon-treated sepals. The color of the treated part of sepals changed very slowly. In about six to seven weeks the sepals turned pale yellow to yellow in color (Fig. 2, b). In the autumn, the treated plants dried up without any noticeable change in color.

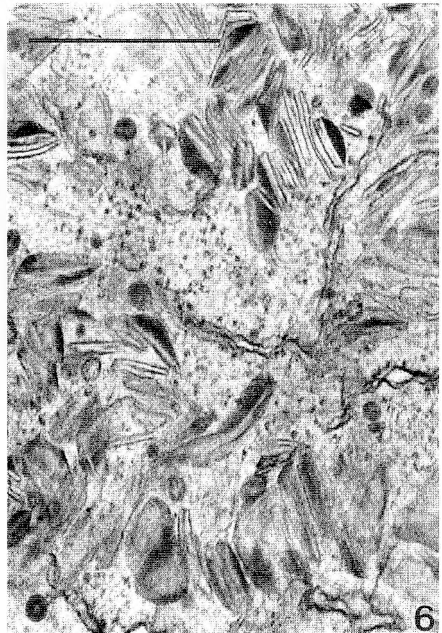
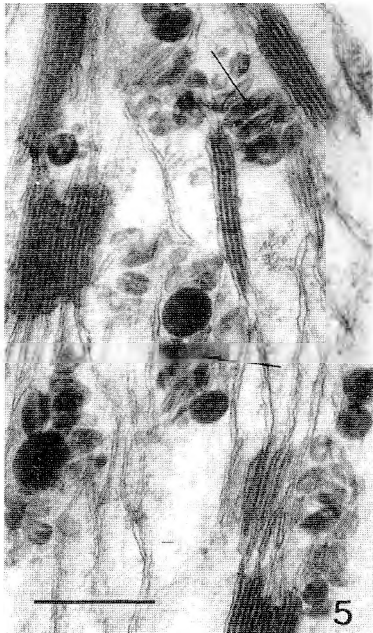
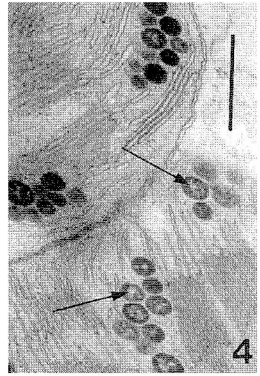
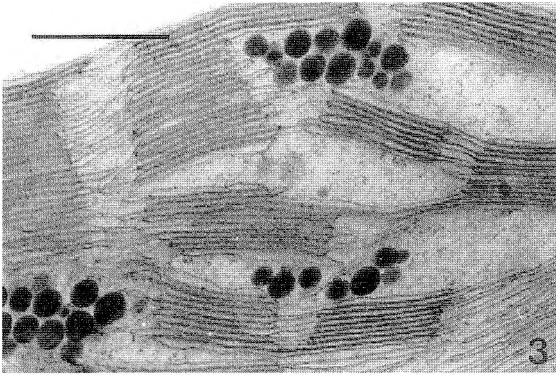
Light microscopy

Control. Light microscopy showed that the green sepals contained typical chloroplasts oval in shape. The red sepals of mature fruit had large and intensely red, mostly spindle-shaped, chromoplasts, which appeared with weak birefringence and dichroitic areas under the polarizing microscope.

Norflurazon-treated sepals. In yellow sepals, about 6 weeks after the beginning of the treatment, only spherical chromoplasts were visible. Light microscopy showed that their color was pale yellow; no particular internal structure could be observed. Birefringence and dichroitic areas under the polarizing microscope were absent.

Electron microscopy

Control. Green sepals of immature fruit contained typical chloroplasts with grana consisting of numerous thylakoids (Fig. 3). Sometimes the number of grana-thylakoids was more than 30. In the dense stroma numerous ribosomes were visible. Plastoglobules, 0.1 to 0.5 µm in diameter, were always in aggregations. Starch grains were rare and very small.



The change in the tissue color corresponded to the gradual disappearance of grana-thylakoids, and accumulation of numerous plastoglobules. At the beginning, the plastoglobules were regularly round in shape and uniformly osmiophilic (Fig. 3). Later, in some of them a light zone appeared, probably due to the beginning of crystallization (Fig. 4). The results of this process were numerous crystalloids growing in the plastoglobules (Figs. 5, 6). Each plastoglobule contained several crystalloids arranged mostly in parallel. In general, the crystalloids remained connected with the remnants of plastoglobules (Fig. 7). The crystalloids were clearly isolated from the stroma by some kind of membrane (Fig. 7). Three-dimensionally considered, the crystalloids were probably platelet-shaped: on the ultrathin sections their dimensions were various - thickness from 10 to 30 nm and length from 0.1 to 1 μm . Sometimes the crystalloids had V-shaped forms with a constant angle of about 110° .

Among the crystalloids, it was possible to find single tubules of about 20 nm in diameter. Connections of tubules with plastoglobules or crystalloids were not found (Fig. 7).

Norflurazon-treated sepals. In the first 2 to 4 weeks after the herbicide treatment, the sepals, as in control plants, contained chloroplasts with typical grana and numerous plastoglobules arranged in large aggregates (Fig. 8). Starch grains were also present. During the subsequent weeks the grana disappeared and the number and the dimensions of the plastoglobules increased. Only long single thylakoids were observed (Fig. 9). The stroma was dense and contained numerous ribosomes. Sometimes the ribosomes were placed on the thylakoids. Relatively numerous nucleoids were visible as translucent areas among the thylakoids and plastoglobules (Fig. 9).

Yellow sepals, 6–7 weeks after treatment, contained chromoplasts which were oval in shape. The whole organelle was filled with numerous plastoglobules of about 0.2 μm in diameter. Their osmiophilicity was lower than in the control chromoplasts (Fig. 10). Only small remnants of single thylakoids were present. The stroma was coarsely-grained and without clearly visible ribosomes.

Pigment analysis

Control. The pattern of pigments in green sepals was similar to that in normal green leaves, although the amount of total carotenoids was somewhat higher as compared to typical leaves. Red sepals from mature fruit contained only carotenoids in very high concentrations (3.3 $\text{mg}\cdot\text{g}^{-1}\text{fr.wt.}$). The majority of these

Figs. 3–7. Differentiation of chromoplasts in sepals of control plants of *Physalis alkekengi*. Bars = 0.5 μm .

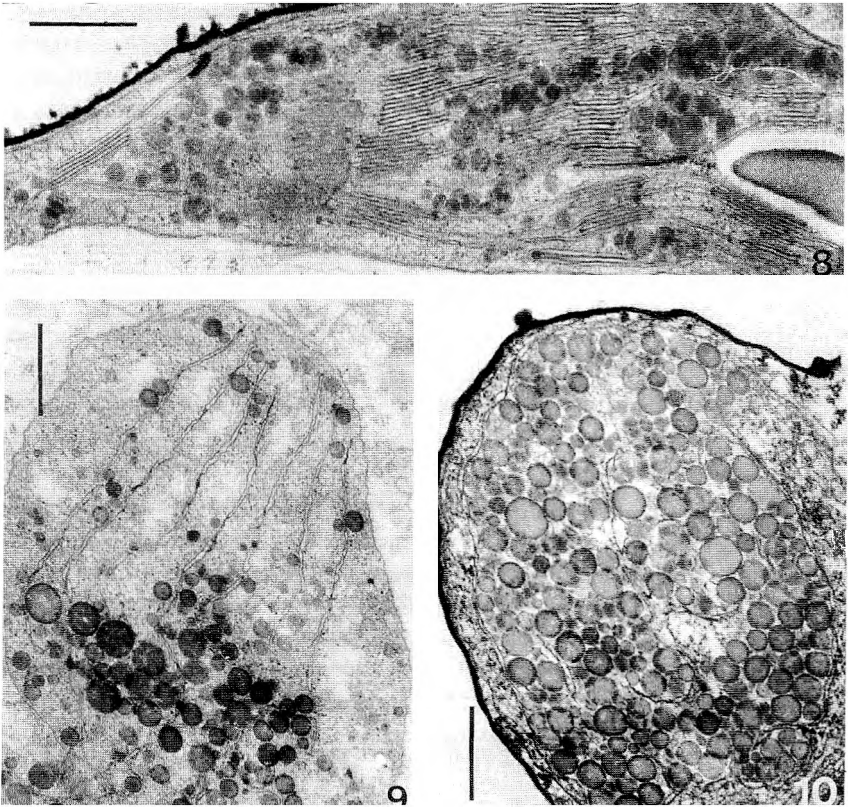
Fig. 3. Part of chloroplast with groups of plastoglobules from green sepal.

Fig. 4. Parts of chloroplasts from yellow-green sepal. Translucent areas in the plastoglobules indicate beginning crystal formation (arrows).

Fig. 5. Part of chromoplast from orange sepal. Small crystalloids (arrows) form in the plastoglobules among the remnants of grana and single thylakoids.

Fig. 6. Part of chromoplast from red sepal. The formation of the crystalloids in the plastoglobules is complete. Only remnants of single thylakoids are present.

Fig. 7. Chromoplast from red sepal. The whole chromoplast is filled with numerous small crystalloids inside the plastoglobules. Single tubules are present (arrowheads).



Figs. 8–10. Plastids from norflurazon-treated sepal. Bars = 0.5 μm .

Fig. 8. Chloro-chromoplast from yellow sepal. The thylakoid system is well preserved and large accumulations of plastoglobules are present.

Fig. 9. Plastid in yellow-white sepal. Among single thylakoids are numerous plastoglobules.

Fig. 10. Plastids from mature yellow-white sepals. The whole plastid is filled with numerous plastoglobules and only a few single membranes remain.

carotenoids consisted of β -carotene (84.4 %). Thin-layer chromatography indicated the presence of a carotenoid (15.6 % of total amount of carotenoids) with a smaller R_f value (about 0.75). According to the results of SIMPSON et al. (1978), this could be zeaxanthin dipalmitate.

Norflurazon-treated sepals. Yellow treated sepals contained only carotenoids (without any traces of chlorophylls), but their concentration was about 20 times lower ($0.105 \text{ mg}\cdot\text{g}^{-1}$ fr.wt.) than in control sepals. The ratio between β -carotene and the putative zeaxanthin dipalmitate was similar. In contrast to the controls, the treated sepals contained lutein, even though in very small amounts.

Discussion

According to the type of carotenoid-bearing structures, chromoplasts are classified in five main groups (for reviews see: SITTE et al. 1980 and CAMARA et

al. 1995). As shown in the present study, the sepals of *Physalis alkekengi* contain chromoplasts of the crystalline type; according to CAMARA et al. (1995) they could be classified as crystalline chromoplasts containing small crystals of β -carotene.

Simultaneously with the formation of crystalloids in the *Physalis alkekengi* chromoplasts, rapid synthesis of β -carotene occurred, reaching more than 84 % of the total carotenoids in the mature sepals. This suggests the β -carotene nature of the small crystalloids.

The small crystalloids of *Physalis alkekengi* chromoplasts were morphologically different from the large crystals found e.g. in carrot roots or daffodil flowers (KUHN et al. 1969, KUHN 1970, HLOUŠEK-RADOJČIĆ and LJUBEŠIĆ 1988). The formation of large β -carotene crystals starts in the lumen of the thylakoids. The small crystalloids from *Physalis alkekengi* chromoplasts never have connections with thylakoids or any other membranes, although they are separated from the hydrophilic cytosol by some kind of membrane. The small crystalloids were in direct contact with the remnants of plastoglobules during the whole period of chromoplast development.

The shape of the small *Physalis* crystalloids is still unclear. Electron micrographs indicate that the crystalloids could be flat plates. However, based on ultra-thin sections, it is not possible to determine if these flat plates are isodiametric or elongated. Their direct contact with round plastoglobules suggests the shape of isodiametric plates. To estimate the real shape of the small crystalloids, they should be isolated.

It is known that norflurazon indirectly inhibits the biosynthesis of β -carotene by blocking the phytoene-to-phytofluene transition (FROSCHE et al. 1979). The effect of norflurazon on the differentiation of chromoplasts in the sepals of *Physalis alkekengi* suggests connections between the accumulation of large amounts of β -carotene and the formation of crystalline structures in the chromoplasts. When the biosynthesis of β -carotene is inhibited, large accumulations of plastoglobules form. A similar effect of norflurazon is observed in the chromoplasts of the corona of daffodil flowers, which contain typical large crystals (HLOUŠEK-RADOJČIĆ and LJUBEŠIĆ 1988), and in the chromoplasts with small crystalloids in the flowers of *Liriodendron tulipifera* (HLOUŠEK and LJUBEŠIĆ 1985).

Analogous findings were described with other bleaching herbicides that affect carotenoid biosynthesis. CTPA (2-(4-chlorophenylthio)ethyl)diethylammonium-chloride treated fruit of *Solanum pseudocapsicum* accumulate plastoglobules and lycopene crystals instead of the typical large β -carotene crystals (SIMPSON et al. 1978). Amitrole (3-amino-1H-1,2,4-triazole) inhibits carotenoid biosynthesis in the petals of *Liriodendron tulipifera* and causes the accumulation of numerous plastoglobules instead of small β -carotene crystals in the chromoplasts (LJUBEŠIĆ and MATIJEVIĆ 1992).

In conclusion, the biosynthesis of large amounts of β -carotene in the chromoplasts results in the formation of crystalline structures. If the concentration of β -carotene is not sufficient for the crystallization, the sequestration of pigments occurs in the lipophilic plastoglobules.

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References

- CAMARA, B., HUGUENEY, P., BOUVIER, F., KUNTZ, M., MONÉGER, R., 1995: Biochemistry and molecular biology of chromoplast development. *Int. Rev. Cytol.* 163, 175–247.
- FROSC S., JABBen M., BERGFELD R., KLEINIG H., MOHR, H., 1979: Inhibition of carotenoid biosynthesis by the herbicide SAN 9789 and its consequence for the action of phytochrome on plastogenesis. *Planta* 145, 497–505.
- HLOUŠEK, A., LJUBEŠIĆ, N., 1985: The effect of SAN 9789 on tulip tree chromoplasts. *Acta Bot. Croat.* 44, 15–18.
- HLOUŠEK-RADOJIĆ, A., LJUBEŠIĆ, N., 1988: The development of daffodil chromoplasts in the presence of herbicides SAN 9789 and SAN 9785. *Z. Naturforsch.* 43c, 418–422.
- KUHN, H., 1970: Chemismus, Struktur und Entstehung der Carotinkriställchen in der Nebenkörone von *Narcissus poeticus* L. Var. "La Riente". *J. Ultrastruct. Res.* 33, 332–355.
- KUHN, H., FREY-WYSSLING, A., STRICKER, P., 1969: Über die Struktur pflanzlicher Karotinkriställchen. *Experientia* 25, 972–973.
- LJUBEŠIĆ, N., MATIJEVIĆ, D., 1992: The effect of amitrole on the pigment composition and ultrastructure of chromoplasts of tulip tree flowers. *Acta Bot. Croat.* 51, 13–19.
- LICHTENTHALER, H. K., 1969: Zur Synthese der lipophilen Plastochinone und Sekundär-carotinoide während der Chromoplastenentwicklung. *Ber. Dtsch. Bot. Ges.* 82, 483–489.
- SELAKOVIĆ, D., LJUBEŠIĆ, N., PREBEG, T., 1996: The influence of carotenoid composition on chromoplast structures. *J. Comp.-Assist. Microsc.* 8, 275–276.
- SIMPSON, D. J., BAQAR, M. R., LEE, T. E., 1978: Chromoplast ultrastructure in fruit of *Solanum pseudocapsicum* and fruit and sepals of *Physalis alkekengi*. *Aust. J. Bot.* 26, 793–806.
- SITTE, P., FALK, H., LIEDVOGEL, B., 1980: Chromoplasts. In: CZYGAN, F.-C. (ed.), *Pigments in Plants*. 2nd ed., 117–148. G. Fischer Verlag, Stuttgart.
- WRISCHER, M., LJUBEŠIĆ, N., PREBEG, T., MAGNUS, V., 1999: The succession of chromoplast structures in *Impatiens noli-tangere* flowers. *Phyton* 39, 49–59.