Bulgarian Journal of Agricultural Science, 21 (No 2) 2015, 300–304
Agricultural Academy

VARIATIONS IN THE CHLOROPLAST ULTRASTRUCTURE IN *IN VITRO*-CULTURED *HYPERICUM* SPP. PLANTS

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Abstract

STEFANOVA, M., D. KOLEVA and T. GANEVA, 2015. Variations in the chloroplast ultrastructure in *in vitro*-cultured *Hypericum* spp. plants. *Bulg. J. Agric. Sci.*, 21: 300–304

Genus *Hypericum* comprises more than 480 species. Some of them have unique therapeutical properties, others are endemic or endangered. These features make the species appropriate objects for biotechnological manipulations. *In vitro* conditions considerably affect organogenesis and hystogenesis of plants subjected to micropropagation. Structural organization of the plastid apparatus in the leaf mesophyll of *in vitro*-cultured plants is an indicator for evaluation of the regeneration potential of the explants.

The subject of the present study is the chloroplast ultrastructure of 7 *in vitro*-cultured *Hypericum* species – *H. perforatum*, *H. humifusum*, *H. kalmianum*, *H. annulatum*, *H. tomentosum*, *H. pulchrum* and *H. rumeliacum*. The aim is to analyze the morphological diversity of these vital for the process of regeneration cell compartments.

TEM-analysis allows us to identify the differences among species at subcellular level of organization in correlation to the *in vitro* conditions. The morphological diversity of chloroplast structure manifests as altered plastid shape, organization of the internal membrane system, and starch content. The chloroplast shape of *H. perforatum*, *H. annulatum*, and *H. pulchrum* is elongated, which is typical for *in vivo* chloroplast. These species also are characterized with properly-structured thylakoid system. However, the grana height, the amount of stromal thylakolds, and the spatial orientation of the entire membrane system can vary. Signs of thylakoid destruction are observed in *H. humifusum*, *H. kalmianum*, *H. tomentosum*, and *H. rumeliacum*. Only three *in vitro*-cultured species (*H. perforatum*, *H. annulatum* and *H. rumeliacum*) develop chloroplasts with large amount of starch.

The results show that the chloroplasts in *H. annulatum* have the most proper structure while the *H. tomentosum* chloroplasts are the most atypical. The great morphological variability in the organization of the plastid apparatus in *Hypericum* species reveals autonomous structural response of each of them to the *in vitro* conditions despite their genetic similarity.

Key words: Hypericum sp., chloroplast ultrastructure, *in vitro,* transmission electron microscopy
Abbreviations: TEM – transmission electron microscopy

Introduction

Structural organization of the chloroplasts, differentiated *in vitro*, is a very informative indicator for the regeneration capability of each species. A lot of TEM-studies examined the morphological variability in the organization of the plastid apparatus of *in vitro*-regenerated plants in search of favorable preconditions for a regular chloroplast structure. Previous ultrastructural studies observed a wide variety in chloroplast structure of *in vitro* plants from different systematic groups (Wetzstein and Sommer, 1982; Sudriá et al., 2001; Majada et al., 2002; Jausoro et al., 2010). It was found that in *in vitro* conditions were quite possible normal chloroplasts to be structured (Laetsch and Stetler, 1965; Sudriá et al., 2001; Kapchina-Toteva and Stoyanova, 2003; Oliveira et al., 2008; Magyar-Tábori et al., 2010; Stoyanova-Koleva et

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al., 2011). Quite often, however, chloroplasts with unusual shape and odd organization of the internal membrane system were noticed (Wetzstein and Sommer, 1982; Lee et al., 1985; Majada et al., 2002; Synková et al., 2003). Also chloroplasts with partially or entirely impaired membrane compartment and varying quantity of plastoglobuli and starch grains were observed (Lamhamedi et al., 2003; Lucchesini et al., 2006; Ladygin et al., 2008; Stefanova et al., 2013). It was shown that any change in the parameters of the culture medium occurred in ultrastructural level in a specific way for each species (Lee et al., 1985; Olmos and Hellín, 1998; Louro et al., 1999; Serret and Trillas, 2000; Oliveira et al., 2008; Stoyanova-Koleva et al., 2011; Stefanova et al., 2013). It has not yet been revealed to what extent the species specificity is a limiting factor for a structuring of the plastid apparatus *in vitro*. Obtaining such information would be possible through a comparative ultrastructural study of genetically related species.

Subject of the present study were 7 *in vitro*-cultured under equal conditions *Hypericum* species – *H. perforatum, H. humifusum, H. kalmianum, H. annulatum, H. tomentosum, H. pulchrum* and *H. rumeliacum*. The aim was to examine the mesophyll chloroplast ultrastructure by TEM and to analyze the morphological diversity of these vital for the process of regeneration cell compartments.

Materials and Methods

Plant material

Stem explants from seven *Hypericum* species (*H. perforatum*, *H. humifusum*, *H. kalmianum*, *H. annulatum*, *H. tomentosum*, *H. pulchrum*, and H. *rumeliacum*) were introduced *in vitro* through direct plant regeneration and cultured on a standard full-strength medium (Murashige and Skoog, 1962), supplemented with 2% (m/v) sucrose and 8.0 $g.dm^{-3}$ agar. The growth conditions were: temperature 22°C, 16-h photoperiod, photosynthetic photon flux density 60 $\mu mol\ m^{-2}\ s^{-1}$ (white fluorescent tubes).

TEM-analysis

After 35 days of cultivation small leaf segments (1–2 mm^2) of fully expanded leaves were taken from the 2nd or 3rd nodes and fixed in 3% (m/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 12 h at 4°C. The leaf segments were post fixed in 1% (m/v) $KMnO_4$ in the same buffer for 2 h at room temperature. After dehydration by increasing concentrations of ethyl alcohol (from 25 to 100%), the samples were embedded in Durcupan (Fluka, Buchs, Switzerland). Ultra thin cross-sections were cut from the palisade parenchyma with Reichert-Jung (Wien, Austria) ultramicrotome and were contrasted with lead citrate (Reynolds, 1963). Observation was performed by JEOL 1200 EX (Tokyo, Japan) electron microscope.

Results

TEM-analysis of the seven *Hypericum* species showed a great diversity in the structural organization of the mesophyll chloroplasts. In cross-section the chloroplasts of *H. perforatum* (Figure 1*A*) were elliptic and the area, occupied by the internal membrane system, was broad. The internal membrane system was structured from a lot of low grana (5–8 thylakoids) and less grana with average height (about 20 thylakoids). The grana were connected with long stromal thylakoids. In the stroma of each chloroplast relatively big starch grains were observed. They consisted of a starch, surrounded by a well-structured enzyme capsule and a very thin sugar zone.

The chloroplasts of *H. humifusum* (Figure 1*B*) in cross-section were semicircular, convex towards the vacuole and flattened towards the cell wall. The internal membrane system was situated at the side of the vacuole. In each chloroplast there was a large peristromium at the side of the cell wall. The majority of the grana were low (up to 10 thylakoids). The stromal thylakoids were long and very dense. There were not starch grains in the stroma.

In cross-section the chloroplasts of *H. kalmianum* (Figure 1*C*) were almost rounded. The spatial orientation of the internal membrane system was atypical. It looked like structured from absolutely separated parts. Each of them had different spatial orientation. The grana were middle high (around 10–15 thylakoids) and very tightly situated. In some of them a fusion of the thylakoids was observed. The stromal thylakoids were short and relatively dense. There were not starch grains in the stroma.

The shape of *H. annulatum* (Figure 1*D*) chloroplasts in transverse section was typical elliptical. The internal membrane system was parallel to the long axis of the chloroplast and consisted of well-organized thylakoids. There was a single small starch grain in the stroma. The thylakoid membranes were typically structured. Almost the entire chloroplast's volume was occupied by high (about 30 thylakoids) and wide grana, connected with few long stromal thylakoids. The starch grains were typically structured with enzyme capsule and zone of sugars.

The chloroplasts of *H. tomentosum* (Figure 1*E*) were oval. The internal membrane system occupied almost the entire volume of the chloroplasts. The thylakoid system was structured from uniformly distributed grana with equal height (around 15–20 thylakoids) and short stromal thyla-

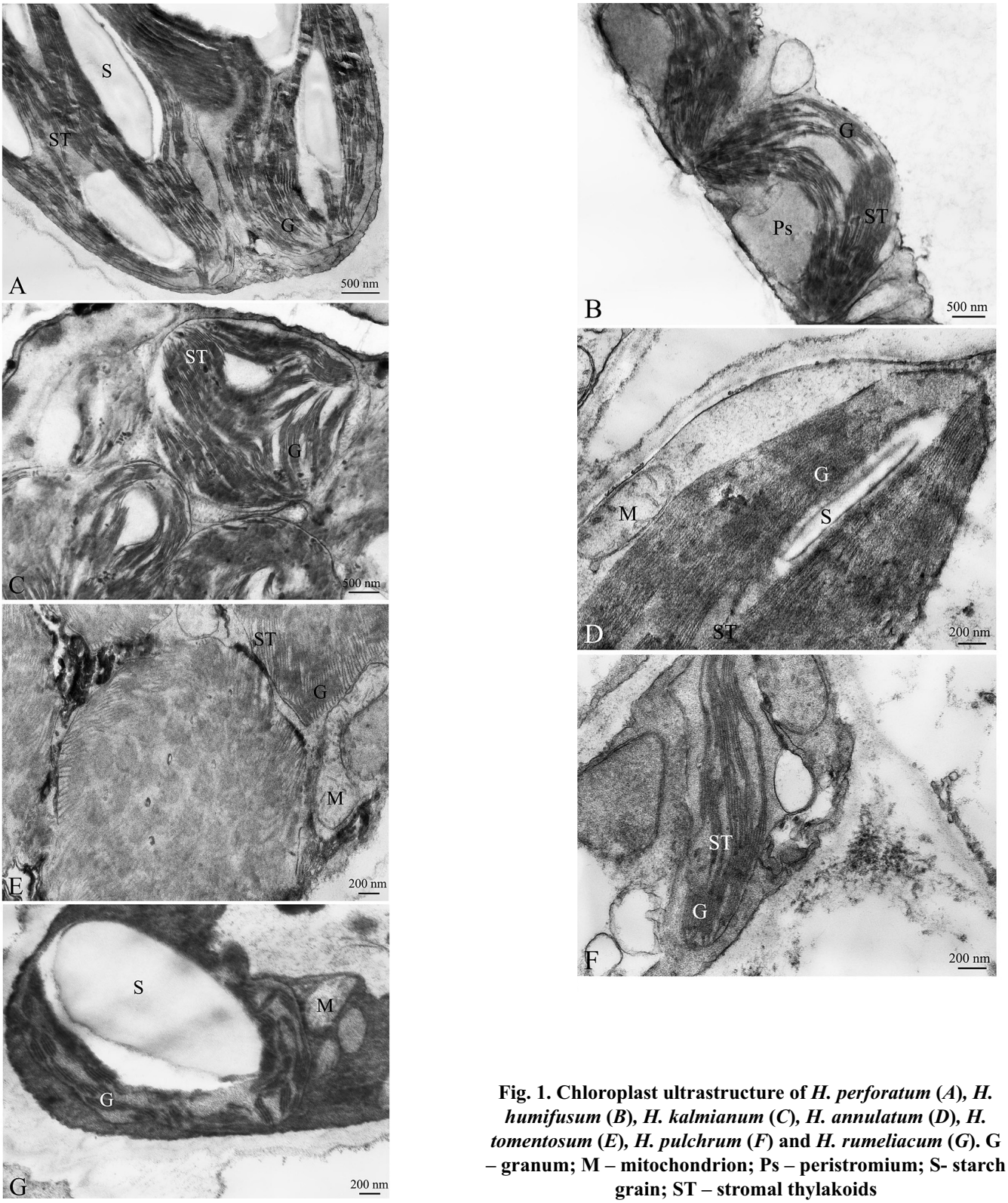

Fig. 1. Chloroplast ultrastructure of *H. perforatum* (*A*), *H. humifusum* (*B*), *H. kalmianum* (*C*), *H. annulatum* (*D*), *H. tomentosum* (*E*), *H. pulchrum* (*F*) and *H. rumeliacum* (*G*). G – granum; M – mitochondrion; Ps – peristromium; S- starch grain; ST – stromal thylakoids

koids. Fusion of the thylakoids in the centrally situated grana was observed. The peripheral thylakoids were well-structured but atypically orientated almost perpendicular to the chloroplast envelope. The atypical spatial orientation of the internal membrane system correlated with the altered shape of the plastids. Starch grains were not present in the stroma. Also peculiar was the shape of the mitochondria. They were very elongated in cross-section and formed large contact area with the chloroplasts.

In the mesophyll of *H. pulchrum* (Figure 1*F*) the chloroplasts were relatively small. In cross-section they were elongated. Their membrane compartment was well-structured. The internal membrane system consisted mostly of low wide grana (5–8 thylakoids) and some grana with middle height (up to 15 thylakoid). The grana were connected with long stromal thylakoid. There were no starch grains in the stroma.

The chloroplasts of *H. rumeliacum* (Figure 1G) were oval which due to one very big starch grain, situated in the center. The internal membrane system occupied a small stromal area in the periphery. The grana were low (around 5 thylakoids) and were built of densely positioned thylakoids. Stromal thylakoids were almost missing. Without any bound between the grana each of them had a different spatial orientation. The starch grains were single, atypically big, with well-structured enzyme capsule and unevenly wide sugar zone.

Discussion

The ultrastructural study of the mesophyll chloroplasts in seven *in vitro*-cultured *Hypericum* species showed morphological diversity in the plastid organization that due to their shape, the stricture of the internal membrane system and the presence or absence of starch. The shape of the chlorplasts in the mesophyll cells of *H. perforatum*, *H. annulatum*, and *H. pulchrum* was typical elliptical while in the rest *Hypericum* species chloroplasts with oval to round shape were formed. The latter resulted in a great variety of spatial orientations of the internal membrane system: like an arc and with broad peristromium in *H. humifusum*; granal and stromal thylakoids with varying spatial orientation in one and the same chloroplast in *H. tomentosum* and *H. rumeliacum*; separated and almost circularly oriented membranes in *H. kalmianum*.

In the studied species the diversity of the structural organization of the internal membrane system was related not only to its spatial orientation, but also to the structure of the thylakoids. Chloroplasts with typically structured thylakoids were formed in *H. perforatum*, *H. annulatum*, and *H. pulchrum in vitro*-plants. However, these chloroplasts clearly differed in the grana height, the length of the stromal thylakolds as well as the quantity of the thylakoids in the organelles. Various grades of a fusion of the granal thylakoids as well as a fragmentation of the stromal thylakoids were found in *in vitro*-chloroplasts from *H. humifusum*, *H. kalmianum, H. tomentosum*, and *H. rumeliacum.* These signs of destruction were most noticeable in *H. tomentosum* plants.

An important structural marker for assessment of the functional state of the regenerated plants, together with the membrane organization of the chloroplasts, is the presence of different amounts of starch in the stroma. In the chloroplasts of *H. perforatum*, *H. annulatum*, and *H. rumeliacum* there was starch in the stroma, but its amount varied: in *H. perforatum* several big starch grains were formed; in *H. rumeliacum* the starch grain was single but very big; in *H. annulatum* the starch grains were one or two, but small. In the chloroplasts of the other four species starch was not observed. The registered diversity in chloroplast organization of *in vitro Hypericum* plants corresponded with previously reported results for other *in vitro*-cultured species (Sudriá, 2001; Majada, 2002; Jausoro, 2010; Stoyanova-Koleva et al., 2012; Stefanova et al., 2013).

Conclusions

Our ultrastructural study found great morphological diversity in the chloroplasts of *in vitro Hypericum* plants. This diversity involved the shape of the chloroplasts, the organization of the internal membrane system and the presence of starch. These structural features were not unique and were observed in other *in vitro*-cultured species. It was obvious that genetically distant species responded in different ways to the specific *in vitro* conditions during micropropagation. Moreover, our ultrastructural study of the seven *Hypericum* species showed that specific *in vitro* conditions could cause considerable diversity in the chloroplast organization of genetically similar species.

Acknowledgements

This work was supported by grants from the Bulgarian Ministry of Education, Youth and Science (DNTC/BGSK-01/2012) and from Slovak Research and Development Agency (APVV SK-BG 0012-10).

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Received August, 2, 2014; accepted for printing January, 8, 2015.