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ISSUES REGARDING THE CHLOROPLAST ULTRASTRUCTURE AND ASSIMILATING PIGMENTS CONTENT IN NORMAL AND HYPERHYDRICE SUGAR BEET (*BETA VULGARIS* L. VAR. SACCHARIFERA) VITROPLANTLET LEAFLETS

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ABSTRACT. Sugar beet plantlet leaflets aged 30 days, obtained from seeds germinated and grown in a greenhouse, or from vitroplantlets, were subjected to ultrastructural and biochemical analysis. In terms of cytology, the foliar mesophyll cells from *in vitro* cultivated plantlets had an increased chloroplasts number than assimilating parenchyma cells of vitroleaflets. More, the vitroplantlet chloroplasts were smaller, had a flatted form, granale and tylacoidale system was poorly represented, but their stroma was rich in starch. Hyperhydrice vitroplantlet leaflets were the most chloroplasts poorest. They were either fusiform or hypertrophies. In some cells they were in a disorganization process, and occasionally, they had not an integrity membrane. In such cells, the chloroplasts are scattered in the myxoplasma, which was formed after the tonoplast disintegration and the cytoplasm was mixed with vacuolar juice. Compared with sugar beet plantlet leaflets, grown in the greenhouse, the hyperhydrice vitroleaflets had 72% less chlorophyll a; in non-hyperhydrice vitroplantlets, the chlorophyll a from leaflets decreased with only 45%; the chlorophyll b content in hyperhydrice vitroplantlets decreased with 50 %, compared to similar parameter that was obtain in greenhouse plantlets; in contrast, at those non-hyperhydrice, the chlorophyll b decreased with only 29.6%. At sugar beet vitrocultivated plantlets, but those non-hyperhydrice, the total green pigments was decreased with 37.2%, compared to that marked in the leaves of plants grown in greenhouse for 30 days, while in hyperhydrice vitroplantlets, the values of this parameter were reduced with 63.3%. Opposite to the carotenoid pigments content recorded of sugar beet plantlets from greenhouse, to normal vitroleaflets the level has decreased with 7%; in contrast, to hyperhydrice vitroplantlets, this parameter decreased with 55%. The experiment has highlighted the profound transformation suffered by sugar beet hyperhydrice vitroplantlets, both in altering terms of the chloroplasts structure and in a fast decrease of the assimilating pigments contents, especially in chlorophyll a.

Keywords: chloroplast, ultrastructure, hyperhydricity, assimilating pigments

INTRODUCTION

For photoautotrophic plants, the specific conditions of the "in vitro" causes the appearance of a morphological transformation and a failure in vitroplantlets tissue to, because of the presence in phytoinoculs vitroculture substrate of an unpolymerised carbohydrate, especially sucrose. This determined conversion of vitroplantlet photoautotrophic metabolisms in one myxotrophic, or even heterotrophic (Cachiţă, 1987; Cachiţă and Crăciun, 1990). In general, in the vitroplantlets foliar limb structure, the palisade tissue is lower, and assimilating parenchyma cells contain a smaller chloroplasts number, than plantlet leaves of same species but growing in the septic conditions; more, in the vitroleaflet chloroplasts stroma are significant starch deposits. On the other hand, vitroleaflets have reduced chlorophyll content, which leads to a low photosynthesis rate (Cachiţă and Ardelean, 2009).

Besides the changes which is observed in the chloroplasts of "normal structured" vitroleaflets, there are situations in which they undergo profound changes, entering their cells in a particular disruption, a phenomenon called hyperhydricity (Živ, 1991; Olmos and Hellin, 1998; Cachiţă and Ardelean, 2004, 2005, 2009). Already in 1987, Rugini and colleagues and Dhawan and Bhojwani (1987) stated that in hyperhydrice vitroleaflets, the chloroplast granas are poorly developed. Also, in the hyperhydricity case, Cachiţă and Crăciun (1990) - at vitroleaflet foliar mesophyll cells of carnations, forsythia, or Chrysanthemums – and Petruş and Cachiţă (2008) at petunia, reported that, in comparison with similar cells of normal vitroplantlets, the size was much increased, their walls become thin and sinuous. In this leaflet, the chloroplasts had a tumefied issue, part of them, showing a disorganized structure, in their stroma there the vacuolization. Sometimes, the chloroplast vacuolization start from a bottom of there, and, gradually, in finally, is a split of their (Cachiţă, 1987). During the hyperhydrice leaflet foliar mesophyll cells degradation, the tonoplast - somewhere - there are in currently desagregations and cytoplasm mix with vacuolar juice, in cells train is a myxoplasm.

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In the hyperhydrice leaflets chloroplasts of pepper vitroplantlets, Fontes and collaborators (1999) have described, they to, the tylacoids disruption phenomenon and the presence of a decreased grana number; however, they have reported an intense accumulation of starch granules in the plastidial stroma and a decrease plastoglobes number, or even their absence in chloroplasts.

MATERIALS AND METHODS

The plant material which was subject for ultrastructural studies and assimilating pigments determination consisted in normal leaflet, taken from „vivo", or from vitroplantlets of sugar beet. In all cases, the cultures were at 30 days from their initiation. The vitrocultures were applied on a base medium (MB) Murashige - Skoog (MS) (1962), without growth regulators. We used caulinar and uninodal apexes, as phytoinoculs, taken from vitroplantlets. In the case of hyperhydrice leaflets, the sugar beet vitrocultures were applied to the same type of MS culture medium, but with the addition of 2.5 mg/l benzyl adenine (BA), a cytokinine that triggered hyperhydricity. As control were used sugar beet leaflets from plantlets obtained from seeds germination in greenhouse, in pots with peat substrate type. Quantities of assimilating pigments which was recorded at this experimental variant were considered the reference, as 100%. Generally, the temperature in the greenhouse and growth room was 25ºC ± 2 - 4ºC; greenhouse crops have been under natural lighting and the vitrocultures illumination was done with fluorescent light, white with 1700 lx intensity and 16 hours/day photoperiod.

Assimilating pigments determination from leafs, respectively chlorophylls a, b and carotenoid pigments was done by extracting them in N, N-dimethylformamide (DMF) 99.9% Merck solution, according to the method developed by Moran and Porath (1980) .

Assimilating pigment quantitative values were obtained by using the absorption coefficients by Welburn (1994), namely:

Formula I - chlorophyll a (μg/gsp)=11.65 A_{664} - 2.69 A_{647} × v/sp;

Formula II - chlorophyll b (μg/gsp) = 20.8 A_{647} - 3.14 A_{664} × v/sp;

Formula III - carotenoids (μg/gsp) = (1000 A_{480} - 1.28 chlorophyll a - 56.7 chlorophyll b)/245 v/sp.

where:

A_{480} - the extinctions read with 480 nm filter, to assess the content in carotenoid pigments;

A_{647} - the extinctions read with 647 nm filter, for the determination of the chlorophyll b;

A_{664} - the extinctions read with 664 nm filter, for determination of chlorophyll content;

v - ml solvent used;

sp - mg of plant material used for extraction/sample;

chlorophyll a and chlorophyll b - mg quantity calculated with the formulas I and II.

Total amount of chlorophyllian pigments was calculated by summing chlorophyll a and b quantities; adding to this figure, the values of the carotenoid pigments was obtain total assimilating pigments extracted from under analysis plant material.

Pigment extractions were made from fresh plant material. Thus, 0.50 g of foliar limb were mortared in 2.5 ml dimethylformamide (DMF), the resulting suspension was kept in the dark, at 4 º C temperature, for 72 hours, then the supernatant was decanted and it served to determine the assimilating pigments quantity, by samples photometration with a spectrophotometer type Spekol 11, Carl Zeiss Jena, using specific filters, previous mentioned. Calibration device was made with the DMF solution. The data obtained were processed statistically by test *t*.

In order to study the ultrastructural aspects of the mesophyll of foliar limb were taken leaflet fragments, which were fixed and processed according to the transmission electron microscopy specific techniques (Hayat, 2000). Fixation was in 2.7% glutaraldehyde for one hour, after which fragments were postfix in 2% osmic acid and dehydrated in increasing concentration of acetone baths; later, the vegetal samples were included in EPONE 812. The section of sample locks was performed with a Leica UC_6 microtome, and the contrastation has been made with lead citrate and uranyl acetate solutions. Preparations were examined with transmission electron microscopy Jeol JEM1010 and images were photographed with a brand digital camera Mega View III CCD. Later, photographs were processed through Corel Photo Paint 12.

RESULTS AND DISCUSSIONS

At normal sugar beet vitroleaflets, microscopic observations made to the preparations obtained from foliar mesophyll, generally showed a similar anatomy with those plants which are 30 days age and cultivated in greenhouse (compared to Fig. 1 A and Fig. 2 A). However, to vitroleaflets, the intercellular spaces were higher and sometimes they have been transformed into lacunas. To the non-hyperhydrice vitroleaflets vacuolar juice was noted the presence of some tiny, solitary or associated in conglomerates formations (Fig. 2 B and C) with unknown nature, but with an electrondense texture. After Cachiţă and Crăciun (1990) they may be liposomes, such as phospholipids, which adsorb anthocyans in their structures.

Regarding the chloroplasts, a first difference between optical microscopy images, seen in cross sections practiced by the foliar limb of sugar beet leaflets provided from "in vivo" or "in vitro" cultivated plants, was that of their *number*/mesophylian cell. Thus, in vitroleaflets foliar mesophyll cells (especially at hyperhydrice plantlets), the chloroplasts number was lower (compared to Fig. 1 A and B, with images of Fig. 2 and 3, A and B) than that found in similar tissue cells harvested from the greenhouse plantlets. More, in vitroleaflets cells, the chloroplast were more oblong (fusiform) and flatten, with starch deposits in the

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stroma. On the other hand, the chloroplasts *size* of foliar mesophyll of vitroleaflets was three times smaller than that of similar plastids present in plantlet leaflets increased ”in vivo”. Otherwise, at non-hyperhydrice leaflets, the electron-microscopic examinations - made with transmission electronic microscope - have not revealed other issues other than those mentioned above.

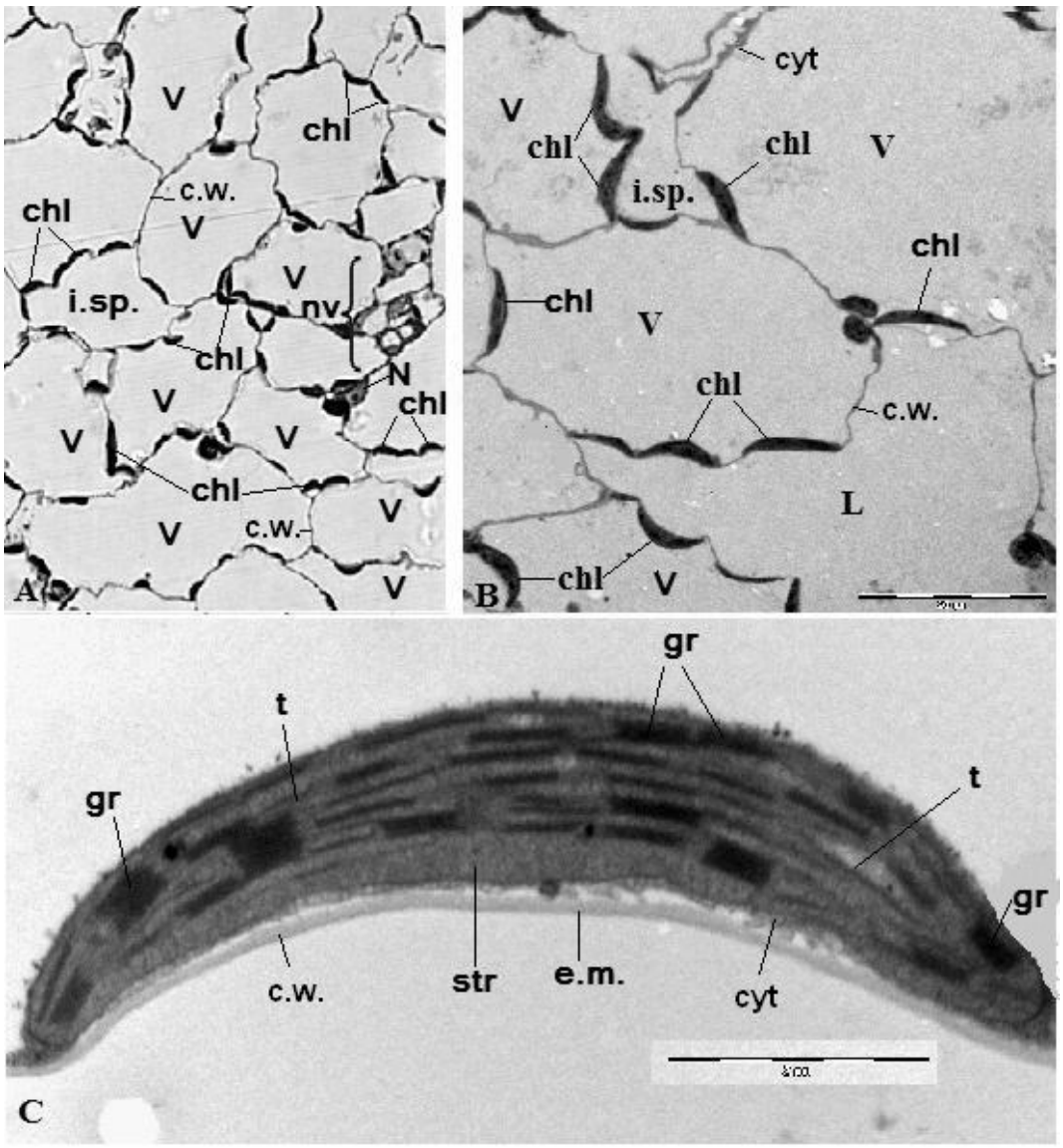

Fig. 1 Aspects of optical microscopy (A) and with transmission electron microscopy (B and C), caught in the foliar mesophyll of sugar beet (*Beta vulgaris* L. var. saccharifera) zygotic plantlet leaflets, grown in natural conditions (where: chl – chloroplasts; c.w. – cellular wall; gr – grana; i.sp. – intercellular space; L – lacuna; N – nucleus; nv – nervure; t – tylacoids; str – stroma; V – vacuole) [A – optical microscopy image - 100x; B and C – transmission electronic microscopy imagines - bare means 20 µm (B) and 2 µm (C) (C- chloroplast –ultrastructural detail)]

Foliar mesophyll of hyperhydrice leaflets was lacunars, between assimilating parenchyma cells there were large intercellular space (true air lacunas), the majority cells were misshapen and had a chaotic disposition. This type of structure corresponds, completely, to that described in literature as being characteristic of the vitroplantlets hyperhydrice tissue (George, 1993).

At hyperhydrice sugar beet vitroplantlets, the chloroplasts were sometimes (even in the same cell) with a hypertrophyate stroma, being disorganized, and is devoid of membrane integrity; chloroplasts, integral or dismantled, often have been scattered in myxoplasm which was formed after tonoplast desagregation and cytoplasm mixing with vacuolar juice (Fig. 3 B and C). Because in hyperhydrice leaflets the chloroplasts structure suffering particular transformation, has been reported in other plant species, vitrocultivated by various other authors (Fontes et al., 1999, Vieth et al., 1983, and by Frank et al., 2004), the sugar beet such issues have been published by us previously (Cachiţă et al., 2008 a-d, and Petrus, 2008).

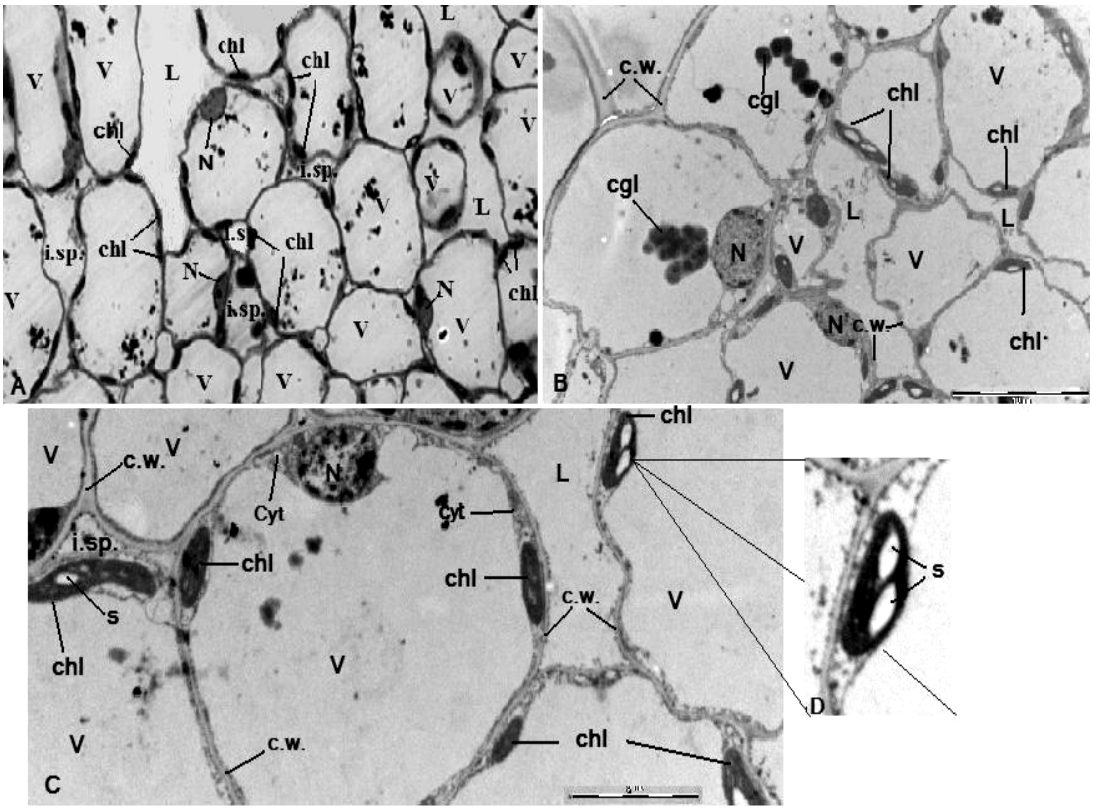

Fig. 2 Aspects of optical microscopy (A) and with transmission electron microscopy (B - C) of normal sugar beet (*Beta vulgaris* L. var. saccharifera) foliar mesophyll cell, grown "in vitro" (where: Cgl – electrondense conglomerates; chl – chloroplasts; cyt – cytoplasm; c.w. – cellular wall; i.sp. – intercellular space; L – lacuna; N – nucleus; s – starch; V – vacuole) [A – optical microscopy image - 100x; B and C – transmission electronic microscopy imagines - bare means 10 µm (B) and 5 µm (C); D – detailed pictures of chloroplast with starch stock in stoma]

Compared with assimilating pigments quantity, determined in sugar beet leaflets of plantlets grown in the greenhouse, which was considered the reference lot (100%), in the hyperhydrice vitroplantlets, the pigments content was considerably lower; so, to this variant, the chlorophyll a level recorded a minus of 72% (Table 1), while in non-hyperhydrice vitroleaflets, its has decreased by only 45%; in terms of chlorophyll b, in normal vitroplantlet leaflets, the content of this pigment decreased by only 29.6% (compared with the respective parameter determined in greenhouse plantlet leaflets), and with 50% at the those hyperhydrice. Consequently, in sugar beet vitroplantlets, but non-hyperhydrice, the total green pigments were reduced by 37.2%, while in hyperhydrice vitroleaflets, the level has decreased by 63.3%. On the other hand, in the normal vitroplantlets, the carotenoids content decreased by 7%, compared to the level reached by these pigments in plantlets from the greenhouse; at hyperhydrice leaflets, this parameter decreased, however, by 55%.

Therefore, assimilating pigment quantities, either chlorophyll a or b, or carotenoids, as it is naturally, were higher o those plantlets leaflets cultivated in greenhouse, compared to their levels identified in normal vitroleaflets, or those hyperhydrice, the minuses which in the case of chlorophylls have proved to be very significant, statistically point of view, and insignificant for carotenoids; in particular, the hyperhydrice vitroleaflets, the pigment contents - all categories - has been considerably diminished.

Experiments carried out under our work revealed especially that the sugar beet plantlets, 30 days aged, both the "vivo" and the "in vitro" (non-hyperhydrice) have held a similar anatomical structure of foliar limbs. However, on hyperhydrice vitroplantlets, the foliar mesophyll suffered profound transformations in terms of form and structure assimilating parenchyma cells, or in case of their low content in the assimilating pigments, especially in chlorophyll a. It is noted, in this context, that in foliar mesophyll cells of sugar beet vitroplantlets has been a smaller number of chloroplasts (and they are smaller sized and fewer granas), which could - partially - explain this phenomenon.

In terms of cytology, in the foliar mesophyll cells of normal (non-hyperhydrice) leaflets, was observed - into vacuoles – some corpuscular electrondense formations, which was absent into greenhouse plantlet leaflets, and into those hyperhydrice, too. This phenomenon could be interpreted as a reaction of these cells to a state of stress. If the case of hyperhydrice leaflet foliar mesophyll cells, these corpuscles are absent because of the vacuole disappearance integrities and training myxoplasm.

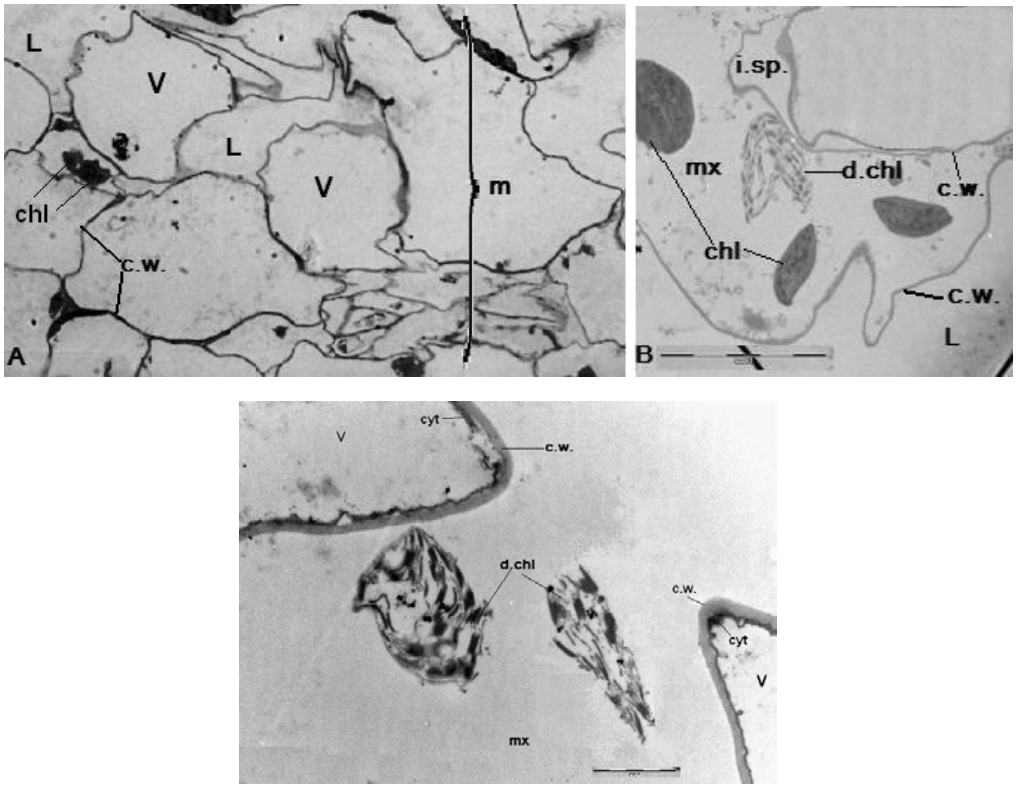

Fig. 3 Aspects of optical microscopy (A) and with transmission electron microscopy (B and C) of hyperhydrice sugar beet (*Beta vulgaris* L. var. saccharifera) foliar mesophyll cell, grown "in vitro" (where: chl – chloroplasts; cyt – cytoplasm; c.w. – cellular wall; d.chl. – disorganized chloroplasts; i.sp. – intercellular space; L – lacuna; m – mesophyll; mx – myxoplasm; V – vacuole) [A – optical microscopy image - 100x; B and C – transmission electronic microscopy imagines - bare means 10 µm (B) and 2 µm (C)]

Table 1

Average values of assimilating pigment quantities determined in extracts prepared from sugar beet (*Beta vulgaris* L. var. saccharifera) plantlet leaflets, grown for 30 days, either "in vivo", in pots, either on Murashige-Skoog (1962) medium, without growth regulators, with the addition of 20 g/l sucrose

Experimental type / Assimilating pigments	Leafs of greenhouse cultivated plantlets (*control*) *average (µg/gsp) ± standard deviation*	*Normal vitroleaflets* from MB-MS without growth regulators		*Hyperhydrice leaflets* from MB-MS+2.5 mg/l BA	
		average (µg/gsp) ± standard deviation	*% toward control*	*average (µg/gsp) ± standard deviation*	*% toward control*
Chlorophyll a	2.0120±0.3785	1.1160±0.2643 **	*55.46%*	0.5676±0.3724 **	*28.2%*
Chlorophyll b	1.9483±0.6485	1.3714±0.4247 **	*70.38%*	0.9687±0.3277 **	*49.7%*
Total chlorophyll pigments	3.9603±0.5698	2.4874±0.3568 **	*62.8%*	*1.5363*±0.2567 **	*36.7%*
Carotenoids	10.2176±0.5247	9.4819±0.2268 ns	*92.79%*	4.8643±0.5773 **	*47.6%*
Total assimilating pigments	14.1779±0.4832	11.9693±0.1458 **	*84.42%*	6.4006±0.3659 **	*45.1%*

***Note**: ns – no significant p>0.05; * = p < 0.05; ** = p < 0.01*

CONCLUSIONS

In contrast with sugar beet (*Beta vulgaris* L. var. saccharifera) plantlets, aged 30 days, provided from seed and grown in greenhouse (reference lot), those normal plantlets vitrocultivated on basic medium Murashige-Skoog (1962) solidified, without growth regulators, with 20 g/l sucrose content had a thin foliar mesophyll, with high intercellular spaces; in this cells

was fewer chloroplasts than recorded in mesophyll cells of greenhouse plantlets. The chloroplasts of vitroplantlet mesophyllian cells were three times lower than those of greenhouse plantlets; also, they had a flatten form and had starch deposits in their stroma. The chlorophyllian pigments quantity which was determined in normal vitroleaflets foliar limb was significantly lower compared with that identified in the plantlets lot provided from greenhouse. More, the palisade tissue cell chloroplast stromas of this do not contain starch.

In hyperhydrice vitroplantlets leaflets foliar mesophyll cells were identified either chloroplasts flatten or volume increased, but with a content being disorganization; they were scattered in vacuolar juice mixed with cytoplasm (myxoplasm), a mixture resulting because the tonoplast disintegration. The hyperhydrice leaflets presented a low content of assimilating pigments, much lower than that determined in sugar beet greenhouse plantlet leaflets, and lower than that recorded in leaflets of „in vitro" nonhyperhydrice plantlets. Thus, comparatively with sugar beet plantlet leaflets grown in the greenhouse, the hyperhydrice vitroleaflets had 72% less chlorophyll a, while in nonhyperhydrice leaflets this parameter decreased by only 45%; the chlorophyll b content in hyperhydrice plantlets decreased by 50%, compared with the greenhouse plantlets; in contrast, to sugar beet nonhyperhydrice leaflets, the chlorophyll b decreased by only 29.6%. At sugar beet vitrocultivated plantlets, but nonhyperhydrice, the total green pigments was decreased by 37.2%, compared to that marked in the plantlets grown in greenhouse for 30 days; at hyperhydrice vitroplantlets the values of this parameter were reduced by 63 3%. In contrast to carotenoid pigment content recorded in sugar beet plantlets from greenhouse, at normal vitroleaflets level of these pigments decreased with 7%, while at hyperhydrice vitroplantlets this parameter decreased by 55%. The experiment showed the profound transformation suffered by hyperhydrice sugar beet plantlets, both in terms of altering the chloroplast structure and a strong decrease of their contents in assimilating pigments, especially in chlorophyll a.

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