BIOLOGIA PLANTARUM 42 (1): 39-48, 1999

Comparison of physiology and anatomy of seedlings and regenerants of sugar beet

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Abstract

Whole sugar beet (*Beta vulgaris* L., cv. Ras poly) plants were grown in the greenhouse from the same seed stock used for an *in vitro* shoot tip culture. *In vitro* produced sugar beet plants exhibited a high content of chlorophylls *a* and *b*, carotene, and total and soluble sugars. On the other hand, total protein content of *in vivo* plants was higher than that of *in vitro* plants. No differences were found by SDS-PAGE analysis in the nature and contents of soluble proteins of *in vitro* propagated plants and greenhouse-grown plants. Surfaces of epidermal cells were larger and palisade and spongy parenchyma tissues were thicker in leaves of regenerants than in leaves of seedlings. Vascular tissues in leaf petioles in regenerants were flat and more differentiated than in seedlings. Closed and undeveloped stomata were found on the abaxial leaf surface of regenerants, whereas in seedlings the stomata were open.

Additional key words: *Beta vulgaris*, carotene, chlorophyll, leaf anatomy, proteins, stomata, sugars.

Introduction

Sugar beet, *Beta vulgaris* L., is the most important sucrose-producing crop in temperate regions of the world. Plant cell and tissue culture techniques are important for improving the genetic and breeding work with crop plants (Cocking and Riley 1981). Many studies on micropropagation of sugar beet confirmed that *in vitro* produced plants were genetically identical (Mezei *et al.* 1990) and no somaclonal variation was detected by Zhong *et al.* (1993). On the other hand, Svirshchevskaya (1991) stated that various genotypes of sugar beet responded differently to culture *in vitro*, and the regenerants showed somaclonal variation.

Received 25 March 1998, *accepted* 20 August 1998.
Abbreviations: BAP - 6-benzylaminopurine; IBA - 3-indolebutyric acid; 2iP - N-isopentenyl-aminopurine; NAA - α-naphthaleneacetic acid; SDS-PAGE - sodium dedocyl sulphate-polyacrylamide gel electrophoresis.
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The effect of the growth medium composition, atmosphere in the culture vessel and irradiation on proliferation, morphogenesis and anatomy of different plant species grown *in vitro* was studied, *e.g.*, by Murashige (1974) and Maene and Debergh (1987). Ultrastructural characteristics of cells from normal and habituated calli was studied by Crèvecoeur *et al.* (1992). The anatomy of the *in vitro* grown "minibeets" was compared to that of *in vivo* sugar beet plants (Toldi *et al.* 1994).

This paper compares different parameters of *in vitro* and naturally grown sugar beet plants. It focused on leaf and petiole anatomy including stomata development and pigment, sugar and protein contents.

Materials and methods

Seed sterilization and *in vitro* germination: Seeds of sugar beet (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima*) cv. Ras poly were kindly provided by Sugar Crops Research Institute of Agriculture Research Centre, Giza, Egypt. They were surface sterilized in 70 % ethanol for 2 min and 30 % *Clorox* (containing 5.25 % sodium hypochlorite) for 25 min, then finally washed four times with distilled sterilized water. Seeds were soaked in distilled sterilized water overnight. For obtaining seedlings, five seeds were placed in 250 cm^3 flasks containing 50 cm^3 MS basal medium (Murashige and Skoog 1962) without any supplementation of growth regulators. The pH of the media was adjusted to 5.8 using 1 M NaOH or HCl, then autoclaved at 121 °C and a pressure of 1.2 kg cm^{-2} for 20 min. Cultures were incubated in a growth chamber at temperature of 25 ± 2 °C, and 16-h photoperiod (irradiance of about 25 µmol(photon) m^{-2} s^{-1} provided by cool white fluorescent lamps).

***In vitro* cultivation of regenerants:** Shoot tips obtained from aseptically grown sugar beet seedlings (15-d-old) were cultured on MS medium supplemented with 0.25 mg dm^{-3} NAA and 1.00 mg dm^{-3} BAP (regeneration medium). Within 10 - 15 d of culture multiple shoots of explanted shoot tips were developed (Fig. 1*A*). After 30 d individual shoots were transferred to MS medium containing 1.00 mg dm^{-3} NAA and 0.25 mg dm^{-3} BAP (multiplication medium) (Fig. 1*B*). After 8 weeks shoots were transferred onto MS medium supplemented with 3.00 mg dm^{-3} IBA and 0.02 mg dm^{-3} 2iP (rooting medium). Rooted plantlets were transferred into pots with a mixture of sand and peatmoss (1:1) and incubated in the growth chamber for one month (Fig. 1C). The obtained plantlets were transplanted *ex vitro* into pots containing garden soil in the greenhouse (for detail see Rady 1997). This procedure allows the rapid and large scale propagation of sugar beet plants, and due to the absence of a callus phase, which is believed to increase the risk of genome destabilization (D'Amato 1985), this pathway of regeneration would appear suitable for this study.

***Ex vitro* and naturally grown plants:** Sugar beet plants produced *in vitro* were transferred into pots in the greenhouse. From the same seed stock used for *in vitro* culture the plants were grown in the greenhouse for the whole life span. After six months naturally grown plants were compared with those produced *in vitro*.

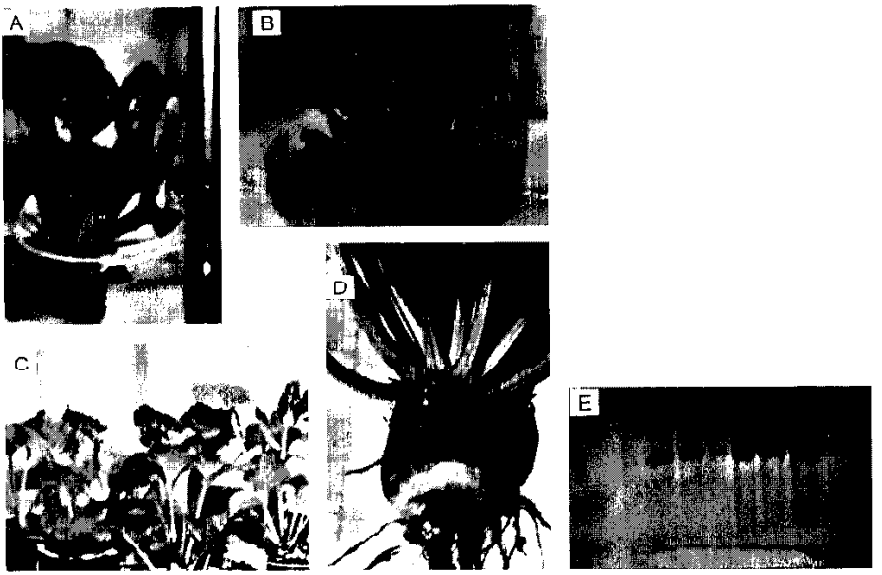

Fig. 1. *A* - shoot proliferation from cultured shoot tips of sugar beet cv. Ras poly, grown on MS medium supplemented with 0.25 mg dm^{-3} NAA and 1.00 mg dm^{-3} BAP; *B* - multiple shoot formation after 8-weeks cultivation on MS medium supplemented with 1.00 mg dm^{-3} NAA and 0.25 mg dm^{-3} BAP; *C* - sugar beet plants during acclimation to *ex vitro* conditions; *D* - roots of *in vitro* produced sugar beet after acclimation; *E* - SDS-PAGE protein patterns of *in vitro* produced plants (lanes 1,2,3) and *in vivo* plants (lanes 4,5,6) (*from left to right*).

Pigments, saccharide and protein contents: The contents of chlorophyll *a* and *b*, and carotene were determined in fresh samples according to the method of Sarić *et al.* (1967). Total saccharide content was determined colorimetrically at 490 nm according to the method described by Herbert *et al.* (1971). Soluble sugars was measured by hand refractometer. For protein extraction 1 g of the fresh tissue was homogenized in 1 cm^3 of sodium phosphate buffer (pH 6.8), centrifuged at 11 400 *g* for 10 min. Total soluble proteins in the supernatant were assayed as described by Bradford (1976). Proteins extracted from leaves of *in vitro* and *in vivo* sugar beet plants were subjected to SDS-PAGE according to Laemmli (1970) using 10 % acrylamide in the separating gel and 3 % in the stacking gel. Samples each of 0.016 cm^3 containing 30 μg protein in equivolume of the extraction and denaturation buffers were denatured for 3 min in boiling water bath, cooled, centrifuged for few seconds at 11 400 *g*, and 0.015 cm^3 were applied per well. The separation was carried out on EC mini gel unit at 60 V for 4 h. Gels were stained with Coomassie brilliant blue (R-250) and destained with 40 % methanol in 10 % acetic acid.

Anatomical studies: Leaf tissue (4 - 6 leaves) from 1-month-old seedlings, germinated aseptically on MS basal medium and 1-month-old *in vitro* regenerants grown on MS basal medium supplemented with 25 mg dm^{-3} NAA + 1.0 mg dm^{-3} BAP and incubated in the same growth chamber was used. For transverse sections samples were taken from the middle parts of the leaves and petioles, killed and fixed in F.A.A. (formalin, acetic acid and alcohol; 90:5:5), dehydrated in ascending concentration of ethyl alcohol according to Johanson (1940), then cleared by soaking in series of xylene absolute ethanol, and embedded in paraffin wax (M.P. 55 - 58 °C). Using a rotary microtome, serial cross sections (15 - 20 μm) were obtained and stained with crystal violet - erythrosine combination (Jackson 1926) and mounted in Canada Balsam. Direct microscopic examinations (*Nikon* light microscope and *Nikon* camera *Fx-35*) were carried out on leaf epidermal strips prepared from upper and lower surfaces of leaf blade to examine stomatal configuration and epidermal cell walls.

Results and discussion

Chlorophyll and carotene contents: Plants grown in the greenhouse showed a lower contents of chlorophyll *a* and *b*, and carotene than *in vitro* produced plants (Table 1). The high chlorophyll content in *in vitro* grown plants might enable high photosynthetic rates when entering in the acclimatization (Saebo *et al.* 1995). In this respect, Krstić *et al.* (1996) reported that chlorophyll and carotenoid contents in calluses derived from hypocotyl, cotyledon and ovule explants of sugar beet cultured *in vitro* showed significant differences. Hypocotyl derived callus had the highest chlorophyll *a+b* content (338 mg kg^{-1}), while cotyledon derived callus had the highest carotenoid content (190 mg kg^{-1}). However, the pigment contents in their calluses were significantly lower than in leaves of sugar beet plants.

Saccharide content: Plants grown in the greenhouse showed a slight lower content in total saccharide content than *in vitro* produced plants (Table 1). The same trend was observed with soluble saccharide contents of sugar beet roots.

Table 1. Chlorophyll, carotene, total and soluble saccharide, and total protein contents of *in vitro* produced sugar beet plants (initiated from *in vitro* shoot tip culture and transferred to the greenhouse) and greenhouse grown plants (initiated from seeds). Means + SE, $n = 5$.

Parameter	*In vitro* produced plants	Greenhouse grown plants
Chlorophyll *a* [mg g^{-1}(f.m.)]	1.268 ± 0.021	0.591 ± 0.022
Chlorophyll *b* [mg g^{-1}(f.m.)]	0.633 ± 0.016	0.277 ± 0.012
Carotene [mg g^{-1}(f.m.)]	0.401 ± 0.020	0.271 ± 0.020
Total saccharides [mg g^{-1}(d.m.)]	192.6 ± 1.452	182.0 ± 1.520
Total proteins [mg g^{-1}(f.m.)]	1.994 ± 0.095	2.156 ± 0.073

Protein content: Total protein content of plants grown in the greenhouse was higher than that of *in vitro* produced plants (Table 1). The protein electrophoretic pattern of physiologically mature *in vitro* produced plants was similar to the pattern produced under *in vivo* conditions and no detectable difference was observed. On the other hand, Ultrika *et al.* (1993) analyzed the protein profiles of different embryogenic cell lines of Norway spruce and found that several proteins are characteristic for specific cell lines.

Leaf anatomy of *in vitro* grown regenerants and aseptically germinated seedlings of sugar beet was evaluated after one month of growth under laboratory conditions. Transverse sections of leaves from both seedlings (Fig. 2*A,C*) and regenerants (Fig. 2*B,D*) showed that upper and lower epidermes consist of one layer of cells which differ in size and shape (cubic, rounded and elongated). Epidermal cells of seedlings appeared to be compact, smaller with thick cell walls and have a thick layer of cuticle, whereas it appeared elongated with thinner cell walls and a thin layer of cuticle in *in vitro* regenerants. A slight increase in thickness of both upper and lower surface of epidermal cells were recognized in seedlings rather than in *in vitro* regenerants (Table 2). In this respect, Pospíšilová *et al.* (1997) reported that under very high humidity and low irradiance of most plantlets grown *in vitro*, the development of cuticle and functional stomata is retarded and so efficient regulation of gas exchange does not operate.

The mesophyll tissue of both seedlings and regenerants consists of one layer of parallel slightly elongated palisade cells below the upper epidermal layer and a spongy tissue of rounded parenchyma cells with small air cavities. Palisade cells of seedlings appeared smaller, compact and contain more chloroplasts, whereas those of regenerants appeared bigger and contain less chloroplasts (not counted) (Fig. 2*C,D*). Thickness of palisade layer of regenerants were greater than that of seedlings (Table 2). In this respect, Reuther (1988) observed less palisade tissue in *in vitro* grown leaves of *Spathiphyllum floribundum* and *Rosa rugosa* compared to leaves grown *in vivo*. Spongy parenchyma of both sugar beet seedlings and regenerants were represented by 4 - 5 layers of parenchyma cells. In regenerants the cells were bigger, thinner walled, less in chloroplasts and with more intercellular spaces than in seedlings. Spongy parenchyma of *in vitro* regenerants was also thicker than in seedlings (Table 2).

Flatted and wider midrib zone was observed in the transverse sections of leaves of regenerants (Fig. 2*B*), whereas it was compact in shape and smaller in size in the leaves of seedlings (Fig. 2*A*). Vascular tissues (phloem, cambium and xylem) of *in vitro* regenerants appeared to increase in width and consisted of three vascular bundles each very close to the others with less differentiation in vascular elements (Fig. 2*B*). Xylem vessel rows in each bundle ranged from 2 - 3 except in the middle vascular bundle which consists of 5 rows, and each row consisted of 1 - 3 xylem vessels with protoxylem directed upward and metaxylem downwards. Small patches of phloem and cambium were also recognized in each bundle. However, in seedlings a clearly distinguished well differentiated one vascular bundle with xylem vessels consisted of 8 - 9 rows with 3 - 6 xylem vessels in each row was observed.

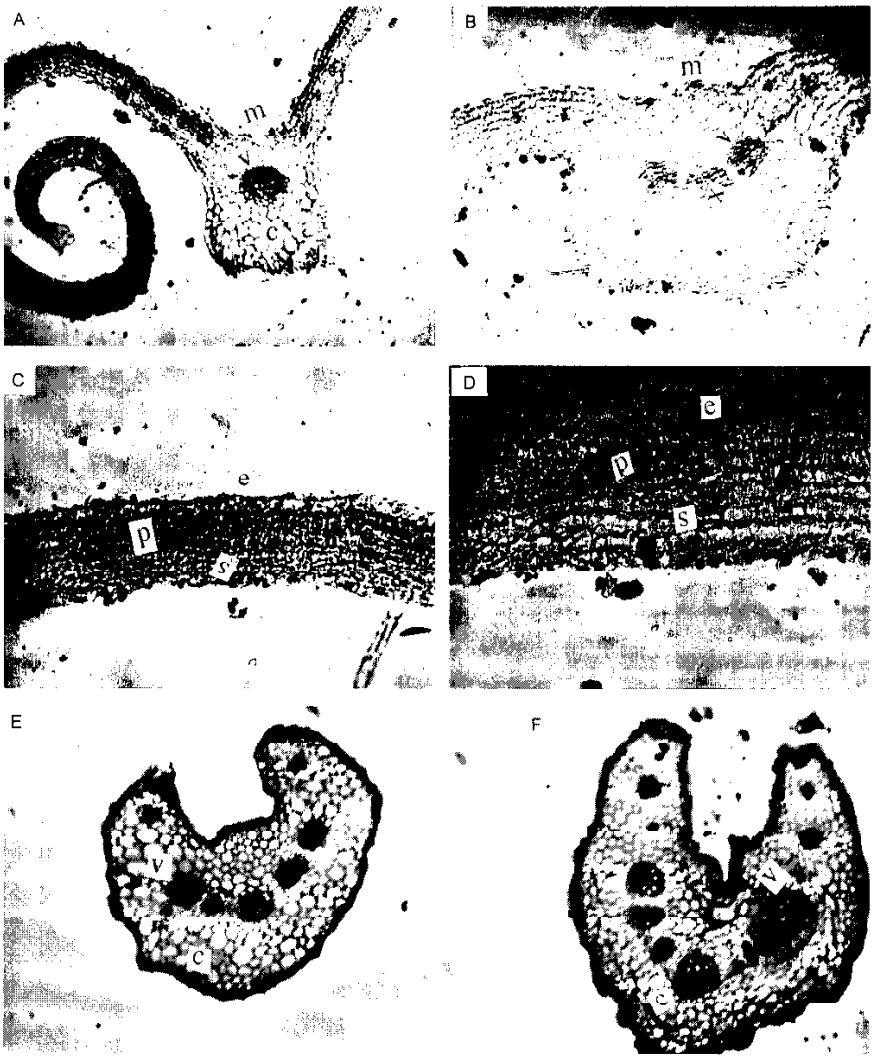

Fig. 2. Transverse sections of leaves and petioles of aseptically grown seedlings (*A,C,E*) and *in vitro* developing regenerants (*B,D,F*) of *Beta vulgaris* L. (×20). c - collenchyma cells, e - epidermal layer, m - midrib zone, p - palisade cells, s - spongy tissue, v - vascular bundles, x - xylem vessels.

Collenchymatous cells behind the vascular bundles were bigger in size and consisted of 14 layers in regenerants, whereas they were smaller in size and compact in shape and consists of 9 layers in seedlings.

Transverse sections of leaf petiole of both *in vitro* developing regenerants and seedlings of sugar beet, appeared triangular in shape and contain several large and some smaller vascular bundles which arranged in a semicircle shape. More rounded, compact, separated and less differentiated vascular bundles were observed in seedlings (Fig. 2*E,F*). In regenerants, the petioles were flat with more differentiated vascular bundles which were surrounded by larger parenchymatous cells.

The above mentioned differences in leaf anatomy may be due to origin of shoots and environmental conditions (*e.g.*, culture medium contain growth regulators which stimulate shoots for rapid growth and multiplication). In this connection, it is worthy to be mentioned that there are great differences in leaf anatomy of other plant species produced by tissue culture techniques and plants grown *ex vitro* from seeds (Grout 1975, Grout and Aston 1978, Brainerd *et al.* 1981, Wetzstein and Sommer 1982, Wardle and Short 1983, Donnelly *et al.* 1985, and Smith *et al.* 1986).

Table 2. Anatomical characteristics in leaves of *in vitro* grown regenerants and seedlings of *Beta vulgaris* L. Each value represents mean ± SE of 3 sections, 10 readings per each.

Thickness [μm]	Regenerants	Seedlings
Upper epidermis	18.0 ± 1.825	19.5 ± 1.707
Lower epidermis	17.0 ± 1.290	18.0 ± 2.160
Palisade parenchyma	34.3 ± 1.201	25.6 ± 0.880
Spongy parenchyma	145.0 ± 2.886	77.0 ± 1.732
Mesophyll	171.0 ± 1.290	103.5 ± 2.753
Whole leaf	205.5 ± 2.101	133.0 ± 1.290
Midrib zone	557.6 ± 1.452	773.0 ± 1.732

In vitro grown regenerants and seedlings of sugar beet revealed also distinct differences on leaf surface (Fig. 3). Epidermal cells from upper and lower surfaces were larger in regenerants than in seedlings. Slightly sinuous anticlinal walls of leaf epidermal cells were only observed in the lower surface of seedlings (Fig. 3*D*). Stomata of regenerants were ellipsoid, bigger in size, enlarged and elevated whereas stomata of seedlings were depressed, rounded and smaller in size. Closed and undeveloping (arrested) stomata were clearly distinguished in the lower surface of regenerants (Fig. 3*B*). Anomocytic and anisocytic stomata types were predominant in both surfaces. However, tetracytic stomata were only observed in the upper surface of regenerants (Fig. 3*A*). Stomata of seedlings were opened (Fig. 3*C,D*).

Most of the previously published reports on leaf anatomy and leaf development concerned on effect of irradiance, *e.g.*, O'Leary and Knecht (1981) demonstrated that at high irradiance, *in vitro* leaves exhibited significantly higher stomatal densities than *in vivo* leaves. In addition to irradiance, stomatal differentiation can be affected by CO_2 concentration, water relations and hormone levels. On the other hand, Lee *et al.* (1988) reported that irradiance had little effect on leaf surface morphology within

in vivo or *in vitro* groups of sweetgum leaves. Stomata of leaves developed *in vitro* were observed to be consistently open, whereas those of *in vivo* were frequently closed and the adaxial surface of epidermal cells of *in vivo* and *in vitro*-grown leaves had sinuous anticlinal walls. *In vitro*-grown leaf epidermal cells were smaller than seedling epidermal cells.

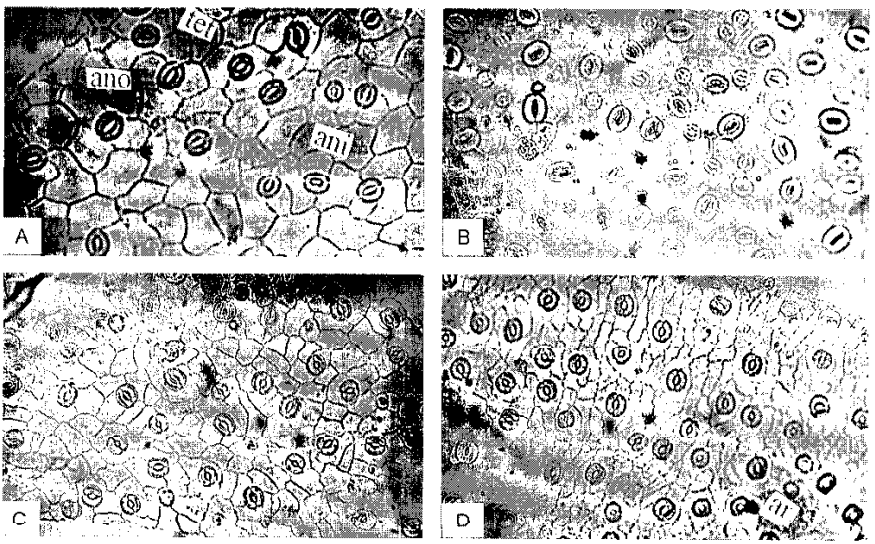

Fig. 3. Adaxial (*A,C*) and abaxial (*B,D*) surfaces of *Beta vulgaris* L., regenerants (*A,B*) and seedlings (*C,D*). Stomata types: ani - anisocytic, ano - anomocytic, ar - arrested, tet - tetracytic (×50).

In conclusion, this study demonstrated a relatively high frequency of variation among *in vitro* and *in vivo* sugar beet plants. The influence of growth regulators in the culture medium can be considered as an important factor for the physiological and anatomical features of sugar beet plants produced in *in vitro*. Furthermore, environmental factors like, changes in photoperiod, increases in temperature, reduction in soil water and nutrients can play a role in controlling gene expression and cellular differentiation *in vivo*. Study of the genetic variation between the *in vitro* and *in vivo* plants using DNA-based markers, such as restriction fragment length polymorphism (RFLP) and (RAPD) fingerprints is needed to confirm the obtained results, revealed by SDS-PAGE, biochemical and histological comparisons.

References

Bradford, M.M.: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. - Ann. Biochem. 72: 248-245, 1976.

Brainerd, K.E., Fuchigami, L.H., Kwiatkowski, S., Clark, C.S.: Leaf anatomy and water stress of aseptically cultured 'Pixy' plum grown under different environments. - HortScience **16**: 173-175, 1981.

Cocking, E.C., Riley, R.: Application of tissue culture and somatic hybridization of plant improvement. - In: Frey, K.J. (ed.): Plant Breeding II. Pp. 85-116. Iowa State Univ. Press, Ames 1981.

Crèvecoeur, M., Hagège, D., Catesson, A. M., Greppin, H., Gaspar, T.: Ultrastructural characteristics of cells from normal and habituated sugar beet calli. - Plant Physiol. Biochem. **30**: 87-95, 1992.

D'Amato, F.: Cytogenetics of plant cell and tissue cultures and their regenerates. - Crit. Rev. Plant Sci. **3**: 73-112, 1985.

Donnelly, D.J., Vidaver, W.E.: Pigment content and gas exchange of red raspberry *in vitro* and *ex vitro*. - J. amer. Soc. hort. Sci. **109**: 177-181, 1984.

Donnelly, D.J., Vidaver, W.E., Lee, K.Y.: The anatomy of tissue cultured red raspberry prior to and after transfer to soil. - Plant Cell Tissue Organ Cult. **4**: 43-50, 1985.

Grout, B.W.W.: Wax development on leaf surfaces of *Brassica oleracea* var. Currawong regenerated from meristem culture. - Plant Sci. Lett. **5**: 401-405, 1975.

Grout, B.W.W., Aston, M.J.: Modified leaf anatomy of cauliflower plantlets regenerated from meristem culture. - Ann. Bot. **42**: 993-995, 1978.

Herbert, D., Phipps, P.J., Strange, R.E.: Determination of total carbohydrate. - Methods Microbiol. **5B**: 209-344, 1971.

Jackson, G.: Crystal violet and erythrosin in plant anatomy. - Stain Technol. **1**: 33-34, 1926.

Johanson, D.A.: Plant Microtechnique. - McGraw Hill Book Co., New York - London 1940.

Krstić, B., Mezei, S., Kovačev, L., Pajević, S.: Chlorophylls and carotenoids in calli of sugar beet genotypes. - Biol. Plant. **38**: 621-624, 1996.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head bacteriophage T4. - Nature **224**: 680-685, 1970.

Lee, N., Wetzstein, H.Y., Sommer, H.E.: Quantum flux density effects on the anatomy and surface morphology of *in vitro* and *in vivo* developed sweetgum leaves. - J. amer. Soc. hort. Sci. **113**: 167-171, 1988.

Maene, L.J., Debergh, P.C.: Optimization of the transfer of tissue cultured shoots to *in vivo* conditions. - Acta Hort. **212**: 335-348, 1987.

Mezei, S., Jelaska, S., Kovacev, L.: Vegetative propagation of sugar beet from floral ramets. - J. Sugar Beet Res. **27** (3-4): 90-96, 1990.

Murashige, T.: Plant propagation through tissue culture. - Annu. Rev. Plant Physiol. **25**: 135-166, 1974.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

O'Leary, J.W., Knecht, G.N.: Elevated CO_2 concentration increases stomata numbers in *Phaseolus vulgaris* leaves. - Bot. Gaz. **142**: 438-441, 1981.

Pospíšilová, J., Čatský, J., Šesták, Z.: Photosynthesis in Plants Cultivated *In Vitro*. - In: Pessarakli, M. (ed.): Handbook of Photosynthesis. Pp. 525-540. Marcel Dekker, New York - Basel - Hong Kong 1997.

Rady, M.R.: *In vitro* multiplication of *Beta vulgaris* L. throughout excised shoot tips. - Biol. Plant. **40**: 515-522, 1997/98.

Reuther, G.: Comparative anatomical and physiological studies with ornamental plants under *in vitro* and greenhouse conditions. Propagation of ornamentals. - Acta Hort. **226**: 91-97, 1988.

Saebo, A., Krekling, T., Appelgren, M.: Light quality affects photosynthesis and leaf anatomy of birch plantlets *in vitro*. - Plant Cell Tissue Organ Cult. **41**: 177-185, 1995.

Sarić, M., Kasztori, R., Ćurić, R., Ćupina, T., Gerić, I.: Chlorophyll determination. - In: Univerzitet u Novom Sadu. Praktikum 12: Fiziologija Biljaka. P. 215. Naučna Knjiga, Beograd 1967.

Smith, M.A.L., Palta, J.P., McCown, B.H.: Comparative anatomy and physiology of microcultured, seedling, and greenhouse-grown Asian white birch. - J. amer. Soc. hort. Sci. **111**: 437-442, 1986.

Svirshchevskaya, A.M.: [Characteristics of sugar beet plants produced in *in vitro* culture.] - In: Sovremennye Metody i Podkhody v Selektsii Rasteniĭ. Pp. 96-101. Kishinev 1991. [In Russ.]

Toldi, O., Gyulai, G., Preininger, E., Várallyay, E., Fári, M., Balázs, E.: Minibeet initiation from derooted sugar beet (*Beta vulgaris* L.) seedling *in vitro*. - Plant Sci. **97**: 217-224, 1994.

Ultrika, E., Lars, H.M., Sara, V.A.: Extracellular proteins in embryogenic suspension cultures of Norway spruce *Picea abies* L. - Physiol. Plant. **88**: 315-321, 1993.

Wardle, K., Short, K.C.: Stomatal response of *in vitro* cultured plantlets. Responses in epidermal strips of chrysanthemum to environmental factors and growth regulators. - Biochem. Physiol. Pflanz. **178**: 619-624, 1983.

Wetzstein, H.Y., Sommer, H.E.: Leaf anatomy of tissue-cultured *Liquidambar styraciflua* (*Hamamelidaceae*) during acclimatization. - Amer. J. Bot. **69**: 1579-1586, 1982.

Zhong, Z.X., Smith, H.G., Thomas, T.H.: Micropropagation of wild beet *Beta maritima* from inflorescence pieces. - Plant Growth Regul. **12**: 53-58, 1993.