Origin and development of secondary somatic embryos in transformed embryogenic cultures of *Medicago sativa*

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Abstract

Non-transformed and transformed embryogenic cultures of alfalfa (*Medicago sativa* L. cv. Zaječarska 83), long-term maintained on growth regulator-free medium, were histologically analyzed. In all examined cultures, somatic embryos at various stages of development were observed and secondary embryos were formed in the cotyledonary, hypocotylary and radicular region of the primary embryos. Detailed histological analysis of the torpedo shape somatic embryo revealed that secondary somatic embryos arose directly from single epidermal cells of hypocotylary axis after an unequal periclinal division. Bipolar proembryos were composed of one smaller cytoplasm rich cell and one larger more vacuolated cell. Further cell division pattern was similar for both non-transformed and transformed embryos. However, multicellular origin of secondary embryos in a direct process and even from callus can not be excluded.

Additional key words: alfalfa, genetic transformation, histological study.

Introduction

Somatic embryogenesis is the process whereby either a single somatic cell or clusters of cells develop into differentiated plants through characteristic embryological stages without fusion of gametes. Somatic embryos can differentiate either indirectly from callus or directly from cells of an organized structure, without an intervening callus phase (Williams and Maheswaran 1986). Direct embryogenesis of unicellular origin reduces the possible appearance of somaclonal variation and occurrence of non-transformed "escape" shoots in genetically transformed cultures.

In the process of secondary or repetitive embryogenesis, new embryos are formed from other somatic embryos in culture. In different *Quercus* species, it has been observed that secondary embryogenesis occurs at the root pole of the primary embryo (Puigderrajols *et al.* 1996), while secondary embryos in walnut arise from cotyledons and hypocotyls of primary embryos (Polito *et al.* 1989). Dos Santos *et al.* (1983) have shown that in alfalfa secondary embryos originate from single epidermal cells, but the authors did not exclude their multicellular origin. Our preliminary cytological studies suggested that at least first and second generation of alfalfa secondary embryos had unicellular origin (Ninković *et al.* 1995).

In this paper, we investigated the origin and development of transformed secondary somatic embryos of *Medicago sativa* in order to establish whether repetitive embryogenesis is a suitable model system for the vegetative propagation of genetically transformed alfalfa plants.

Materials and methods

Plant material: Two transformed and one control non-transformed embryogenic clones of alfalfa (*Medicago sativa* L. cv. Zaječarska 83) that have been maintained in culture for three years were used as starting material. Initial non-transformed embryogenic cultures were obtained from immature zygotic embryos and propagated

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Abbreviations: GFP - green fluorescent protein; *nptII* - neomycin phospho-transferase II; OCII - oryzacistatin II.
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by recurrent embryogenesis (Ninković *et al.* 1998). Single somatic embryos at the cotyledonary stage were used for transformation with *Agrobacterium tumefaciens* strain EHA101 carrying the binary vector pGV-OCII that contains a green fluorescent protein (GFP) gene under the control of CaMV35S promoter, the *nptII* gene under the control of nopaline synthase promoter and the oryza-cistatin (OCII) gene under the control of pin2p promoter (Samac and Smigocki 2003). Inoculation methods and establishment of axenic transformed embryogenic cultures were as described by Ninković *et al.* (1995).

Culture medium and culture conditions: Embryogenic cultures were maintained on a medium containing Murashige and Skoog (1962) mineral salts, B5 vitamins (Gamborg *et al.* 1968), 3 % sucrose and 0.6 % agar (*Torlak*, Belgrade, Serbia and Montenegro). The pH of the medium was adjusted to 5.8 prior to autoclaving at 114 °C for 25 min. The cultures were grown in a temperature controlled chamber at 25 ± 2 °C under an 8-h photoperiod, unless stated differently. Photosynthetic photon flux density of 31 μmol m^{-2} s^{-1} was provided by "white" fluorescent tubes (*Tesla*, Pančevo, Serbia and Montenegro, 65 W, 4500 K).

Histology: For paraffin sections, segments of embryogenic tissue were excised 30 d after the subculture to fresh medium. Isolated material was fixed in FAA (formalin/acetic acid/ethanol 10:5:85), dehydrated in graded ethanol series and embedded in paraffin at 58 °C. Sections (10 μm thick) were stained with Alcian blue and Kernechtrot (nuclear fast red) and photographed using a *Leitz DMRB* photomicroscope (*Leica*, Wetzlar, Germany).

For semi-thin sections, individual somatic embryos at the torpedo stage were isolated from 30-d-old embryogenic cultures and placed on fresh medium under a 16-h photoperiod. At 1 d and 10 d, isolated embryos were collected and fixed in 3 % phosphate-buffered glutaraldehyde, pH 7.2 for 2 h at 4 °C. After a brief wash with buffer, the material was postfixed in 1 % phosphate-buffered OsO_4 for 1 h at 4 °C. The samples were dehydrated in graded ethanol series and embedded in Araldite. Sections (1.5 μm thick) were stained with methylene blue and photographed as above.

Results

Non-transformed and transformed embryogenic alfalfa cultures were maintained for three years on culture media lacking plant growth regulators to enable cyclic proliferation of somatic embryos. Presence of the inserted genes in transformed somatic alfalfa embryos was confirmed by PCR analysis (data not shown).

In basal parts of all cultures callus tissue of different colour and texture was formed and somatic embryos at various stages of development were present (Fig. 1*A*). Non-transformed cultures (clone 3) contained compact green callus with numerous embryos at initial stages of development on its surface. Scarce cotyledonary embryos were mainly fused along their axes and only seldom germinated (Fig. 1*B*). Transformed cultures (clones 4 and 16) contained more embryos at later stages of development, with a green cotyledonary region and a whitish hypocotyl-root part. Apart from well-developed single somatic embryos, embryos fused along their embryonic axes were also frequently present. Germinated embryos with elongated root were mainly characteristic for clone 16 (Fig. 1*C*). Secondary somatic embryos were formed as small green globular structures in the cotyledonary, hypocotylary and radicular region of the primary embryos.

Histological analysis showed that callus tissue of embryogenic cultures consisted of various types of cells: from small meristematic ones to highly vacuolated parenchymatous cells (Fig. 1*D*). In addition, friable callus tissue in transformed cultures was significantly more porous (particularly clone 16) and often contained broad area of necrotic tissue (particularly clone 4, Fig. 1*E*), compared to compact calli that were distinct feature of non-transformed cultures (Fig. 1*F*).

At the periphery of the callus comprised of large vacuolated cells, proembryogenic cell complexes were observed. These multicellular structures gave rise to a number of embryos, probably in the process of cleavage polyembryony (Fig. 1*F*). The presence of embryos at various stages of development (globular, heart-shaped, torpedo and cotyledonary) on the same preparation shows great asynchrony of the developmental process (Figs. 1*E,G,H*). In transformed cultures, somatic embryos at torpedo and cotyledonary stages usually could be easily released from surrounding tissue. Cotyledonary embryos had well developed shoot and root apical meristem, elongated hypocotyl, radicle and clearly differentiated vascular elements (Fig. 1*I*).

Detailed histological analysis of both non-transformed and transformed somatic embryos at the torpedo stage revealed intensive meristematic activity in the superficial layers along the hypocotyledonary region (Fig. 1*E*). Unequal periclinal divisions of single cells were frequently observed one day after transfer of isolated embryos to fresh medium (Fig. 2*A*). After an unequal cell division, a somatic proembryo was formed as a bipolar structure comprised of one smaller cytoplasm rich cell and one larger more vacuolated cell (Fig. 2*A,B*). The smaller initial cell, that was usually apical in this bipolar structure, underwent further divisions forming globular somatic embryo with clearly differentiated protoderm (Fig. 2*C*). The larger cell mainly ceased further divisions so that embryos mostly did not develop suspensor. During next ten days proliferation extended along entire embryo axis, making peripheral layers clearly distinguishable from subjacent tissue (Fig. 2*D*). At the sites of somatic embryo initiation, cells of the subjacent

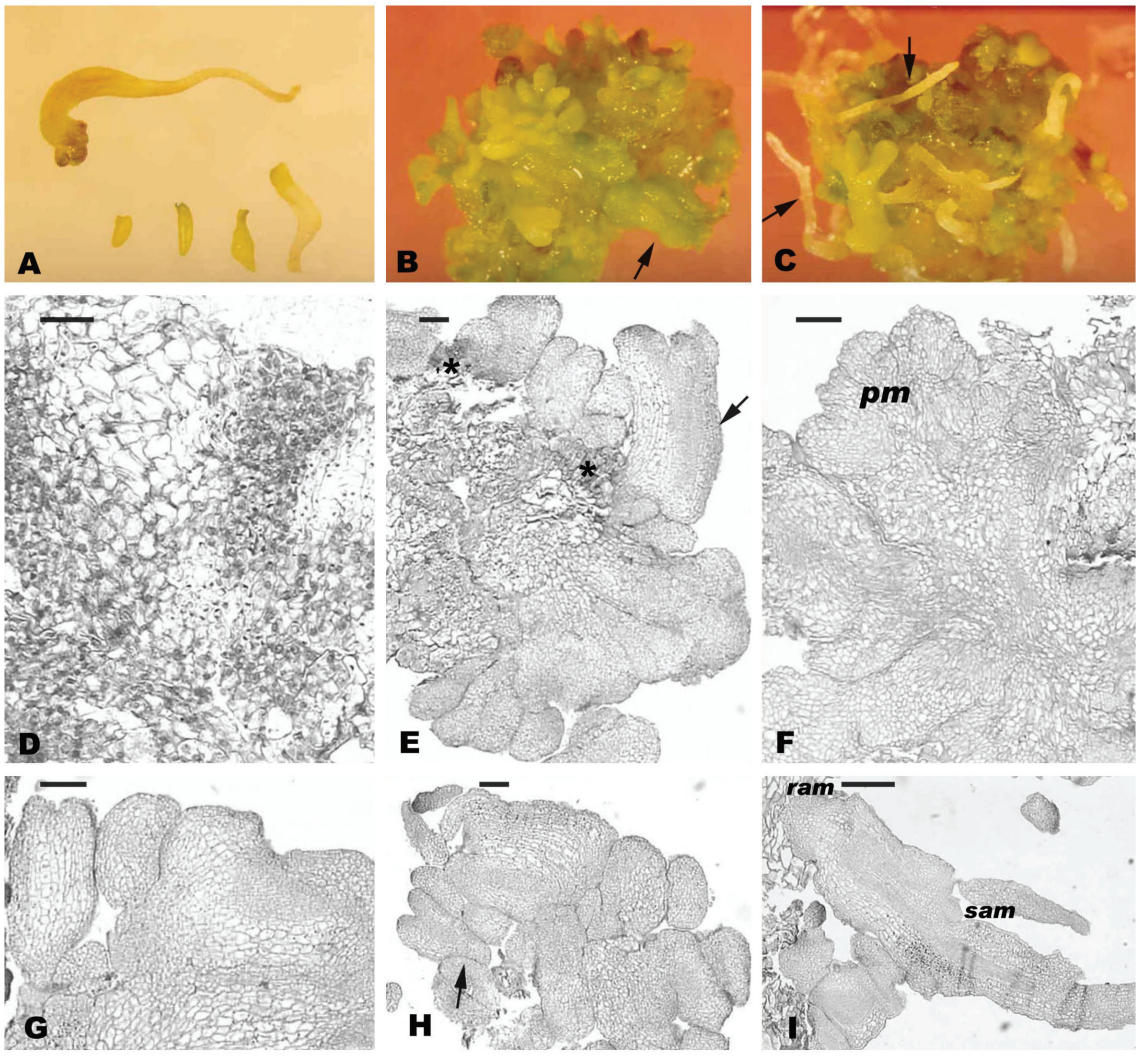

Fig. 1. Morphology and anatomy of alfalfa embryogenic cultures. *A* - somatic embryos at various stages of development; *B* - non-transformed embryogenic culture (clone 3) four weeks after transfer to fresh B5 medium. Note fused embryos at late-torpedo stage (*arrow*); *C* - transformed embryogenic culture (clone 16) four weeks after transfer to fresh B5 medium, containing numerous germinated embryos (*arrows*); *D* - callus tissue in transformed embryogenic culture (clone 16) consisting of various cell types, *bar* = 50 µm; *E* - cross section of transformed embryogenic culture (clone 4) comprising friable callus tissue with pores and necrotic regions (*). Note meristematic activity in the hypocotylary region of cotyledonary somatic embryo (*arrow*), *bar* = 100 µm; *F* - cross section of non-transformed embryogenic culture with compact callus in the center and proembryogenic mass (*pm*) at the periphery, *bar* = 100 µm; *G* - longitudinal section of torpedo stage embryo with adjacent globule in non-transformed embryogenic culture, *bar* = 100 µm; *H* - asynchronous development of somatic embryos in transformed culture. *Arrow* indicates early cotyledonary embryo, which can be easily released from the porous callus tissue, *bar* = 100 µm; *I* - longitudinal section of mature cotyledonary embryo in transformed culture. Note distinctly closed root pole with root apical meristem (*ram*), shoot apical meristem (*sam*) between cotyledons and advanced tissue differentiation: epidermis, primary cortex with numerous starch grains in the upper part of hypocotyl and two procambial bundles branching into cotyledons, *bar* = 200 µm.

layers usually had significantly more starch grains and protein bodies (Fig. 2*B*) compared to the neighbouring cell layers on whose surface there were no signs of cell proliferation.

Globular structures and multicellular proembryogenic complexes of different size and age could be observed along embryo axis, again suggesting that repetitive embryogenesis is an asynchronous process. Even at this early stage, in some embryos regular anticlinal divisions in protoderm were disturbed and at these sites, new somatic embryos were induced (Fig. 2*E*). Besides somatic embryogenesis, proliferation of callus tissue was also observed along hypocotyledonary region. Intensive meristematic activity at the periphery of this non-differentiated tissue probably results in indirect formation of proembryogenic complexes (Fig. 2*F*).

Globular somatic embryos passed through further developmental stages and turned green. During embryo development, transformed somatic embryos appeared to be similar to embryos regenerated from control cultures. In both non-transformed and transformed cultures, some embryos at the cotyledonary stage showed abnormal morphologies, such as hypertrophied appearance and ill-formed cotyledonary ends. Some of the embryos had no visible cotyledons but produced roots from the radicular ends. Morphologically normal cotyledonary embryos usually germinated, but even so their further development was arrested due to emergence of new somatic embryos in the epicotylary region.

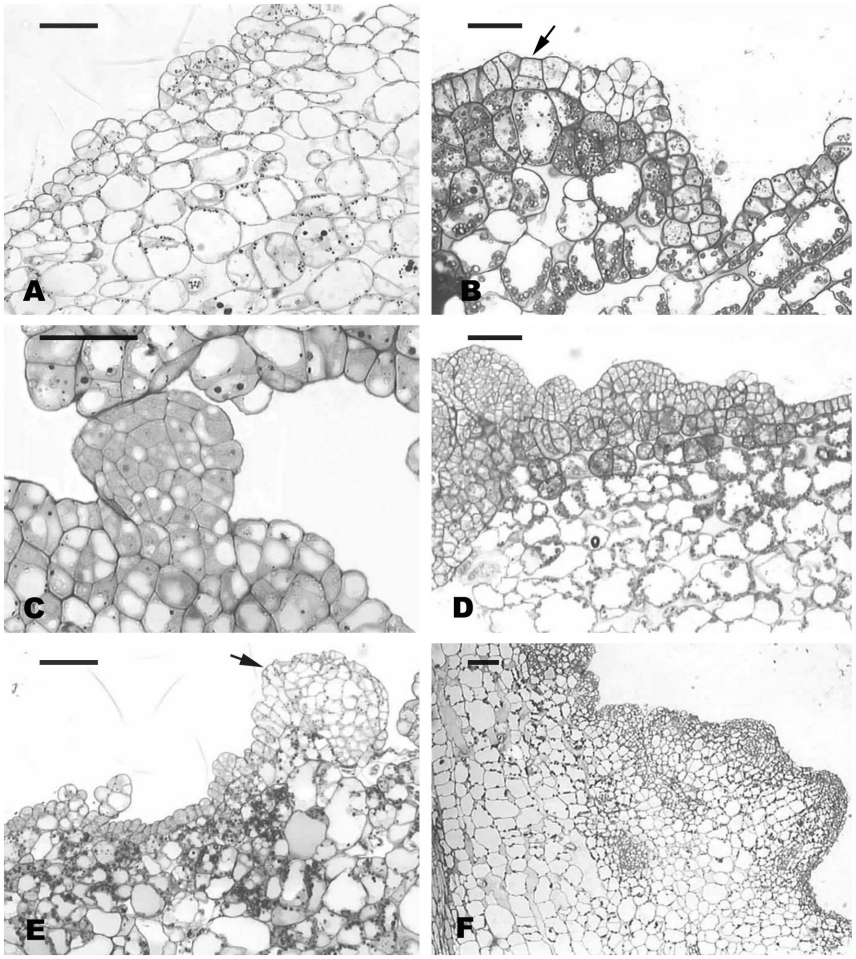

Fig. 2. Histology of secondary somatic embryogenesis. *A, B* - sections of hypocotyl of somatic embryo at the torpedo stage 1 d and 10 d after subculture to fresh medium, respectively. Unequal periclinal divisions of single cells occur in the epidermis; some of them produce opposite orientation of somatic proembryos in maternal tissue explant (*arrow*). Protein bodies are clearly visible in the hypocotyl of early torpedo stage (1 d after subculture, *A*), while later starch grains accumulate in the cells underlying proliferating superficial regions (10 d after subculture, *B*), *bar* = 50 µm; *C* - section of somatic embryo epicotyl 10 d after subculture. One-celled layer of protoderm is differentiated on the surface of the globule, *bar* = 50 µm; *D*, *E*, *F* - section of somatic embryo hypocotyl 10 d after subculture: *D* - globular multicellular proembryogenic complexes along embryo axis, *bar* = 100 µm; *E* - induction of new somatic embryo occurs in the protoderm of globular embryo (*arrow*), *bar* = 50 µm; *F* - densely stained meristematic cells at the periphery of the callus developed on embryo hypocotyl, *bar* = 100 µm. *A, E* - clone 3; *B, C, D, F* - clone 16.

Discussion

Histological analysis of repetitive embryogenesis has revealed that secondary somatic embryos originate directly from epidermal cells of primary alfalfa somatic embryos. Somatic embryos were shown to arise directly from epidermal and subepidermal layers of cotyledonary explants in pigeonpea (Mohan and Krishnamurthy 2002) and of meristematic dome in pea (Griga 2002). Initiation of somatic embryoids directly from superficial cells of the hypocotyl has been reported for immature zygotic embryos of *Brassica campestris* (Maheswaran and

Williams 1986). Sagare *et al.* (1995) reported that in *Cicer arietinum* somatic embryos arose directly from epidermal and subepidermal cells of mature embryo axes, but could also be initiated indirectly with an intervening callus phase from immature embryo axes. This is consistent with our finding that in some cases primary embryo axes developed callus tissue from which new embryos probably arise in the process of cleavage polyembryony, with boundaries between embryos often unclear.

The changes in the position of the division planes in single epidermal cells of hypocotyls and cotyledons, that switch from normal anticlinal to unequal periclinal divisions, could account for unicellular origin of secondary somatic embryos. In direct somatic embryogenesis in *Coffea arabica*, most initial divisions (approximately 90 %) in subepidermal layers of leaf explants were periclinal to leaf surface (Quiroz-Figueroa *et al.* 2002). Dos Santos *et al.* (1983) showed that in the epidermis of cotyledons, hypocotyls and roots of callus-derived plantlets of alfalfa adventitious embryoids often originated from single cells. Our results confirm that the smaller initial was usually apical in this bipolar structure, but could sometimes be found as a basal cell as well. Accordingly, some secondary embryos were most probably attached to the parental tissue by their shoot apical pole.

Multicellular proembryogenic complexes observed along embryo axis probably originated as a result of coordinated growth of a group of embryogenically competent cells on the surface of embryo hypocotyl. These structures were attached to the underlying tissue by broad basis, which may also be considered as an argument for their multicellular origin. Proembryogenic complexes could give rise to two or more secondary embryos in the process of cleavage polyembryony. Cleavage polyembryos could account for fused embryos observed in both non-transformed and transformed cultures.

In both non-transformed and transformed cultures, globular secondary embryos further developed into heart-shaped, torpedo and cotyledonary embryos. Transformed embryos at the cotyledonary stage were mostly fully developed: embryos had closed radicular end and a pair of green cotyledons. In cotton, a majority of transformed somatic embryos at maturity were morphologically abnormal and therefore seldom converted to plantlets (Chaudhary *et al.* 2003). Maturation was also found to be the least efficient stage of succesful plant regeneration from non-transformed somatic embryos of *Chenopodium rubrum* and *Chenopodium murale* (Milivojević *et al.* 2005). Mature alfalfa cotyledonary embryos had low conversion rates to plantlets due to novel cycles of secondary embryogenesis along their axes and radicles. Low frequency of somatic embryo maturation and germination is well known to be the limiting factor for the application of somatic embryogenesis for the improvement of a number of species. An interesting solution to this problem was proposed by Sauer and Wilhelm (2005), as they found that development and maturation of somatic embryos in *Castanea sativa* was stimulated on medium with increased agar concentration.

In conclusion, no apparent difference in origin and histology of non-transformed and transformed secondary alfalfa somatic embryos was observed. Therefore, secondary somatic embryogenesis could serve as a useful method for scale-up production of transformed plants. However, conditions to interrupt repetitive embryogenesis, thus allowing embryo maturation and germination are still to be defined.

References

Chaudhary, B., Kumar, S., Prasad, K.V.S.K., Oinam, G.S., Burma, P.K., Pental, D.: Slow desiccation leads to high-frequency shoot recovery from transformed somatic embryos of cotton (*Gossypium hirsutum* L. cv. Coker 310 FR). - Plant Cell Rep. **21**: 955-960, 2003.

Dos Santos, A.V.P., Cutter, E.G., Davey, M.R.: Origin and development of somatic embryos in *Medicago sativa* L. (alfalfa). - Protoplasma **117**: 107-115, 1983.

Gamborg, O.L., Miller, R.A., Ojima, K.: Nutrient requirements of suspension cultures of soybean root cells. - Exp. Cell Res. **50**: 151-158, 1968.

Griga, M.: Morphology and anatomy of *Pisum sativum* somatic embryos. - Biol. Plant. **45**: 173-182, 2002.

Maheswaran, G., Williams, E.G.: Primary and secondary somatic embryogenesis from immature zygotic embryos of *Brassica campestris*. - J. Plant Physiol. **124**: 455-463, 1986.

Milivojević, S., Mitrović, A., Ćulafić, Lj.: Somatic embryogenesis in *Chenopodium rubrum* and *Chenopodium murale in vitro*. - Biol. Plant. **49**: 35-39, 2005.

Mohan, M.L., Krishnamurthy, K.V.: Somatic embryogenesis and plant regeneration in pigeonpea. - Biol. Plant. **45**: 19-25, 2002.

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

Ninković, S., Miljuš-Djukić, J., Nešković, M.: Genetic transformation of alfalfa somatic embryos and their clonal propagation through repetitive somatic embryogenesis. - Plant Cell Tissue Organ Cult. **42**: 255-260, 1995.

Ninković, S., Miljuš-Đukić, J., Nešković, M.: Genetic engineering of herbicide-resistant alfalfa (*Medicago sativa* L. cv. Zaječarska 83). - In: S. Stamenković (ed.): Proc. of 2nd Balkan Symp. on Field Crops. Vol. 1. Pp. 457-460, Institute of Field and Vegetable Crops, Novi Sad 1998.

Polito, V.S., McGranahan, G., Pinney, K., Leslie, C.: Origin of somatic embryos from repetitively embryogenic cultures of walnut (*Juglans regia* L.): implications for *Agrobacterium*-mediated transformation. - Plant Cell Rep. **8**: 219-221, 1989.

Puigderrajols, P., Fernández-Guijarro, B., Toribio, M., Molinas, M.: Origin and early development of secondary embryos in *Quercus suber* L. - Int. J. Plant Sci. **157**: 674-684, 1996.

Quiroz-Figueroa, F.R., Fuentes-Cerda, C.F.J., Rojas-Herrera, R., Loyola-Vargas, V.M.: Histological studies on the

developmental stages and differentiation of two different somatic embryogenesis systems of *Coffea arabica*. - Plant Cell Rep. **20**: 1141-1149, 2002.
Sagare, A.P., Suhasini, K., Krishnamurthy, K.V.: Histology of somatic embryo initiation and development in chickpea (*Cicer arietinum* L.). - Plant Sci. **109**: 87-93, 1995.
Samac, D.A., Smigocki, A.C.: Expression of oryzacystatin I and II in alfalfa increases resistance to the root-lesion nematode. - Phytopathology **93**: 799-804, 2003.
Sauer, U., Wilhelm, E.: Somatic embryogenesis from ovaries, developing ovules and immature zygotic embryos, and improved embryo development of *Castanea sativa*. - Biol. Plant. **49**: 1-6, 2005.
Williams, E.G., Maheswaran, G.: Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group. - Ann. Bot. **57**: 443-462, 1986.

Fageria, N.K., Baligar, V.C., Clark, R.B.: **Physiology of Crop Production.** - Food Products Press, An Imprint of the Haworth Press, New York - London - Oxford 2006. 345 pp. USD 49.95 (soft), 69.95 (hard). ISBN 978-1-56022-288-0.

In last several decades there is great demand for increasing food production. The present book includes eight chapters that cover extensively both theoretical and practical aspects of crop physiological processes that have profound effects on productions potentials of major agricultural crops.

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The following four chapters discuss crop yield with connection to photosynthesis, source-sink relationships, and carbon dioxide. The impact of global climate change, mainly the still increasing atmospheric CO_2 concentration, and photosynthesis are the main problems discussed (crop and canopy photosynthesis, C_3 and C_4 plants, radiation use efficiency, leaf area index and partitioning of assimilates, respiration during photosynthesis, and management strategies for maximizing photosynthesis).

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