STRUCTURAL CHARACTERISTICS OF *CHRYSANTHEMUM MORIFOLIUM* RAMAT (ROMICA CULTIVAR) REGENERATED *IN VITRO*

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Abstract: The micropropagation of *Chrysanthemum morifolium* Ramat (Romica cultivar), belonging to the collection of “Anastasie Fătu” Botanical Garden from Iasi (Romania) was achieved through tissue culture technique and involved callus induction followed by shoot multiplication, rooting and establishment of plantlets in soil. The purpose of this study was to determine the range of variation in certain structural characters of the vegetative organs of *in vitro* regenerated plants at *Chrysanthemum morifolium* Ramat (Romica cultivar). The material subjected to the comparative anatomical analyses was represented by vegetative organs of the parent plant (PP) and regenerated plant (RP), on mature stage . The density of glandular and non-glandular hair (mm^{-2}) on both leaf surfaces was statistical analysed using “t” test at 0,05 confidence level. Despite the great opportunity of genetic variation in callus cultures, the regenerated plants differ not in their structural appearance from the normal plants.
Key words: *Chrysanthemum morifolium,* callus, organogenesis, anatomy, glandular hair, non-glandular hair.

INTRODUCTION

Chrysanthemum morifolium Ramat is well known, not only as an ornamental plant, but also as an important medicinal plant and a major source of natural products (flavonoids, sesquiterpene lactones, essential oils, triterpene diols and triols) used as pharmaceuticals.

It has been found to possess antibacterial, antifungal, antiviral, antispirochetal and anti-inflammatory activities (Wang *et al.*, 1998, Kishimoto *et al.* Ohimiya, 2006, Ukiya *et al.* 2001, Ukiya *et al.* 2002).

Indirect regeneration of *Chrysanthemum morifolium* Ramat (Romica cultivar) through callus cultures represents an unconventional alternative to preserve, to perpetuate and to exploit the source of raw material for natural compounds.

The present study aims at a detailed histo-anatomical analysis of the vegetative organs (adventitious roots, stem and leaf) of *in vitro* and *in vivo Chrysanthemum morifolium* Ramat (Romica cultivar) plants in order to determine the range of structural variation in the regenerated plant.

MATERIAL AND METHODS

Plant material

The mature *Chrysanthemum morifolium* Ramat (Romica cultivar) plants, belonging to the collection of “Anastasie Fătu” Botanical Garden from Iasi (Romania) were used for initiation of “in vitro” cultures (Vântu, 2005, Vântu, 2006)

Explants sterilization

Stem fragments cutting from the mature plants were sterilized with 3% sodium hypochlorite for 10-15 minutes and again washed thoroughly with sterilized distilled water.

Callus culture

The sterilized stem fragments were cut into rectangular pieces of approximately 5 mm in diameter. Stem explants were implanted apical side down to stimulate the natural flow of auxins and carbohydrates.

The callus cultures were established from stem explants on Murashige and Skoog, (1962) medium, supplemented with 2 mg^{-l} 2, 4-dichlorophenoxyacetic acid and 0,2 mg^{-l} kinetine. Segments of approximately 1 x 1 cm of these calluses were subcultivated on Murashige and Skoog, (1962) medium supplemented with the same combination and concentration of the growth regulators.

Plant regeneration

The callus differentiated into shoots when transferred to MS medium containing 2mg/l kinetine and 0,2 mg/l 2,4 –dichlorophenoxyacetic acid.

The shoots originated from callus tissue were transferred to Murashige and Skoog, (1962) medium without hormones for three months in order to obtain the whole plant (Vântu, 2006)

Histo-anatomical analysis

The material subjected to the comparative anatomical analysis is represented by vegetative organs of the parent plant (PP) and regenerated plant (RP), on mature stage. Adventitious roots, leaves and fragments of stem (cutting from lower, middle and upper level of the plant) were fixed and conserved in 70% ethylic alcohol. Free hand transverse sections were performed using a razor blade. The sections were coloured with ruthenium red and iodine green. The

observations and photomicrograph were done with an Olympus BX51 research microscope equipped with Olympus E-330 photo camera.

Morphometric assessment

For the comparative morphometric assessment between parent plant and regenerated plant (on mature stage), the density of glandular and non-glandular hair (mm^{-2}) on both leaf surface was measured. These characters were analyzed on paradermal stem and leaf sections under 200X magnification using an Olympus BX51 research microscope. For each parameter 10 measurements randomly chosen was made.

Statistical analysis

Data from the morphometric assessment were analyzed by using "t" test (EXCEL) at 0,05 confidence level. Independent samples and unequal variances were assumed.

RESULTS AND DISCUSSIONS

Plant regeneration through callus cultures

The morphogenetical potential has been established to be greater in callus cultures derived from stem explants. The organogenic callus grows rapidly and shows early an extensive organization. The vigorous callus pieces, about 5 mm transferred to fresh medium at intervals of 4 weeks maintain the organogenic callus lines.

The callus differentiated into shoots when transferred to MS medium containing 2mg/l kinetine and 0,2 mg/l 2,4- dichlorophenoxyacetic acid.. After 30 days, green shoots appeared and roots were developed in MS medium with no growth regulators. Root induction on shoot cultures was achieved by subculturing the shoots.

Comparative vegetative anatomy between *in vitro* regenerated and parent plant

In the regenerated plant resulted here by subculturing the shoots, the adventitious root has an endogenous origin and presents primary structure. The stele is of triarch type, showing a multiseriate pericycle and a developed vascular system. The cortex is hypertrophied and do not end with an obviousness endodermis.

According to Toma *et al.* (1985), *in vivo Chrysanthemum morifolium* Ramat (Romica cultivar) plant, the adventitious roots formed on the rhizome passes early from primary to secondary structure, resulted from the activity of both lateral meristems (cambium and phellogen). In the primary structure, the stele is of triarch type and the endodermis has Casparian strips.

The excellence of the root system is a key factor for the success of the acclimatization process (Gonçalves *et al*, 1998). The incomplete vascular connection between shoot and roots of cauliflower rooted *in vitro* resulted in insufficient water translocation to the shoot, endangering the acclimatization of the new plants (Grout & Aston, 1977). The histo-anatomical differences observed here in the adventitious roots formed on the shoots of regenerated plant could change the performance of plant acclimatization.

Despite the stressing conditions of *in vitro* culture media, the stem and leaf of the regenerated *Chrysanthemum morifolium* Ramat (Romica cultivar) plant conserve the structural layout of the parent plant ones.

Along the stem (Fig. 1), from top to base, the passing from primary to secondary structure may be observed. Comparatively with the parent plant, the histological characters of the regenerated plant stem are similar, with small differences: the absence of the cortical hadrocentric vascular bundles, the hypertrophy of the cortical parenchyma, the absence of an obviousness endodermis (see table I and II).

In both parent and regenerated plant, the foliar limb (Fig. 2) has bifacial heterofacial structure, the mesophyll being differentiated in one layer of palisade and 4-5 layers of spongy parenchyma cells. Each lateral vascular bundle is surrounded by a parenchymatous theca.

Both epidermis of *Chrysanthemum morifolium* Ramat (Romica cultivar) leaf in regenerated and parent plant bear apart from stomata, glandular hairs (producing of essential oils) and non-glandular hairs. The foliar limb is amphistomatic, the stomata being of anomocytic type. The glandular hairs have uni- or bicellular gland. The non-glandular hairs are multicellular, with shuttle-like shape on superficial section.

Table I. Comparison between the structure of the upper third of stem in *Chrysanthemum morifolium* Ramat (Romica cultivar) *in vitro* regenerated and parent plant

Stem structural layout (primary structure)	Histological features	
	Regenerated plant (on mature stage)	**Parent plant (on mature stage)**
epidermis	single-layered with stomata, glandular and non-glandular hairs	single-layered with stomata, glandular and non-glandular hairs
cortex	parenchymatous-celllulosic of meatic type	-parenchymatous-celllulosic of meatic type - cortical vascular bundles of hadrocentric type
stele	- collateral vascular bundles(disposed on a circle) + isolated groups of phloem elements - secretory canals (localized in the medullary rays or at the big vascular bundles periphery)	- collateral vascular bundles(disposed on a circle) + isolated groups of phloem elements - secretory canals (localized in the medullary rays or at the big vascular bundles periphery)
pith	parenchymatous-celllulosic of meatic type	parenchymatous-celllulosic of meatic type

Table II. Comparison between the structure of the lower third of stem in *Chrysanthemum morifolium* Ramat (Romica cultivar) *in vitro* regenerated and parent plant

Stem structural layout (secondary structure)	Histological features	
	Regenerated plant (on mature stage)	**Parent plant (on mature stage)**
peridermis	the phellogen is differentiated from the hypodermic layer	the phellogen is differentiated from the hypodermic layer
cortical parenchyma	- meatic type - numerous cells with pericline and anticline division walls	meatic type
endodermis	not evident	Casparyan type
stele	- bundles of secondary phloem (at the outer part) - periphloemic cordons of sclerenchymatous fibers - secondary lignified xylem ring - primary vessels surrounded by cellulosic parenchymatous cells	- bundles of secondary phloem (at the outer part) - periphloemic cordons of sclerenchymatous fibers - secondary lignified xylem ring - primary vessels surrounded by cellulosic parenchymatous cells

	(in the inner part)	(in the inner part)
pith	-parenchymatous –lignified (in the outer part) - parenchymatous-celllulosic of meatic type (in the central part)	- parenchymatous –lignified (in the outer part) - parenchymatous-celllulosic of meatic type (in the central part)

According to Hazarika (2006), the special conditions during in vitro culture results in the formation of plantlets of abnormal morphology, anatomy and physiology. Tissue culture conditions that promote rapid growth and multiplication of shoots often results in the formation of structurally and physiologically abnormal plants.

The results of our histo-anatomical study revealed that in *Chrysanthemum morifolium* Ramat (Romica cultivar), the stressing conditions of *in vitro* culture media and of the confined environment do not affect the structural layout of the vegetative organs.

Comparative morphometric analysis between *in vitro* regenerated and parent plant

The density of glandular hairs was found to be greater in both foliar surfaces of the regenerated plant than in the parent plant ones (Fig. 3). The "t" test indicated significant differences ($P \leq 0,01$) in glandular hair number between regenerated and parent plant.

With respect to the non-glandular hairs, leaves of regenerated plant were more (on upper surface) or less (on lower surface) hairy than those of parent plant (Fig. 4). The "t" test indicated no differences ($P > 0,05$) in non-glandular hair number between regenerated and parent plant.

The leaf surface represents the interface between the plant and the environment. This lead to study the surface of *in vitro* grown plant leaves in comparison to the *in vivo* mother plants (Bandyopadhyay *et al.*, 2004). According to Brutti *et al.* (2002), the trichomes of *in vitro* leaves are poor developed and scanty distributed. Despite this observation our morphometric results revealed no significant differences in non-glandular hairs number between *Chrysanthemum morifolium* Ramat (Romica cultivar) regenerated *in vitro* and parent plant. The density of glandular hair increases in regenerated plant leaves comparatively with the parent plant ones.

The stress in *in vitro* culture may thus contain both destructive and constructive elements: it is a selection factor as well as a driving force for improved resistance and adaptive evolution (Gaspar *et al.*, 2002). The frequency of somaclonal variation would depend on the culture protocol applied during the *in vitro* process, particularly on the hormone composition of the medium and the number of subcultures (Ducos *et al.,* 2003).

Thus, the organogenic regeneration procedure using in our study would not cause destructive structural variations of vegetative organs in *Chrysanthemum morifolium* Ramat (Romica cultivar).

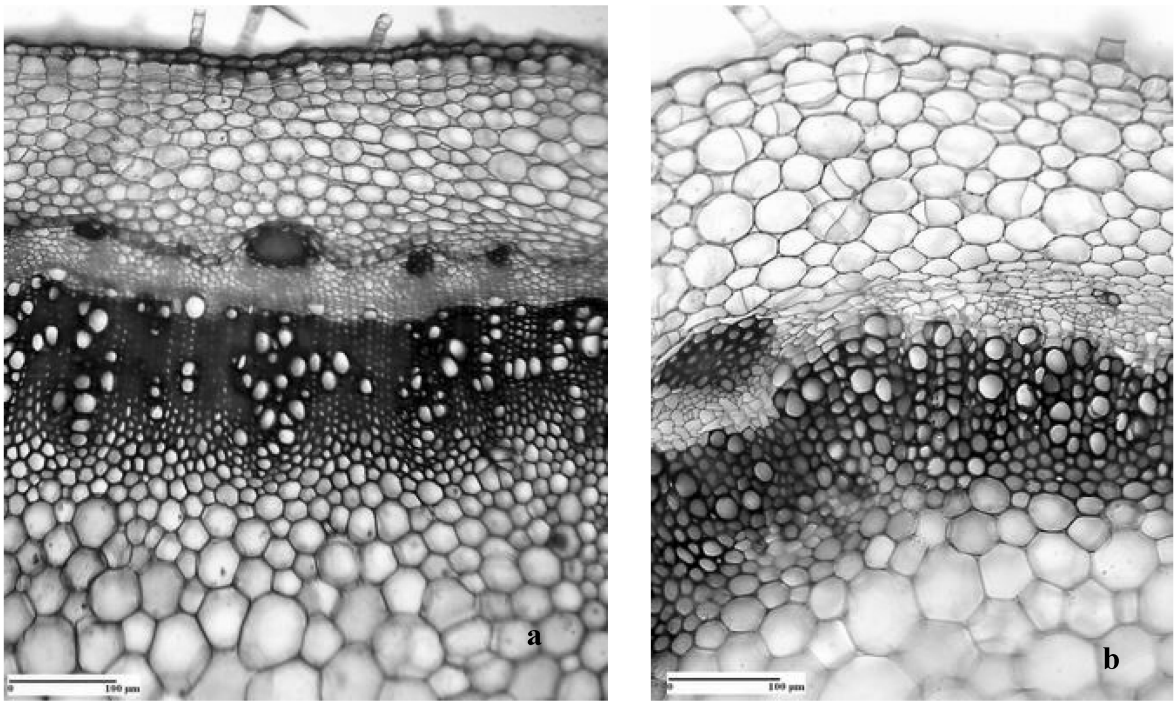


Fig. 1. Cross-section through the lower level of stem in *Chrysanthemum morifolium* Ramat (Romica cultivar) parent plant (**a**) and *in vitro* regenerated plant (**b**).

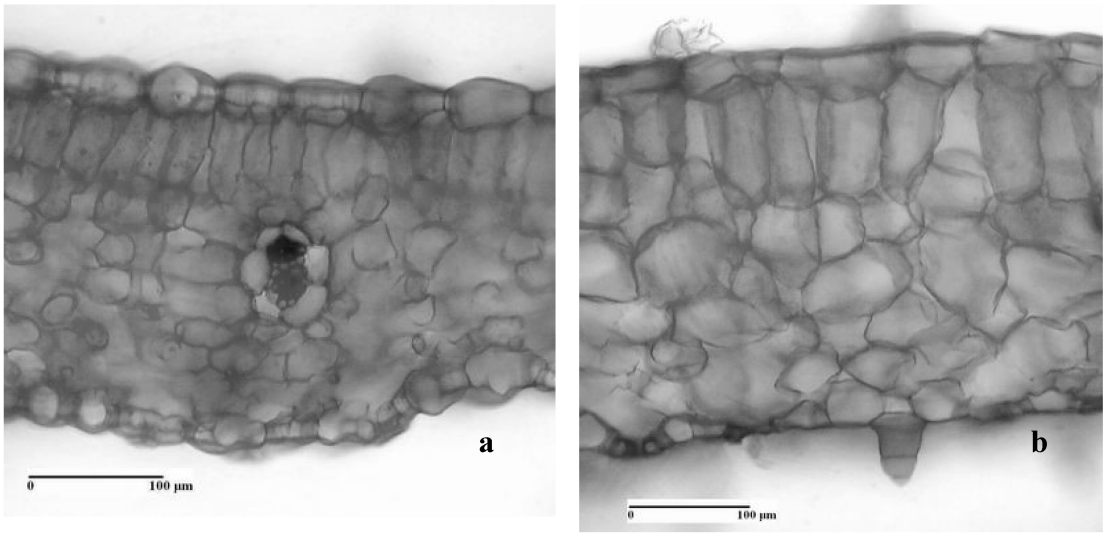
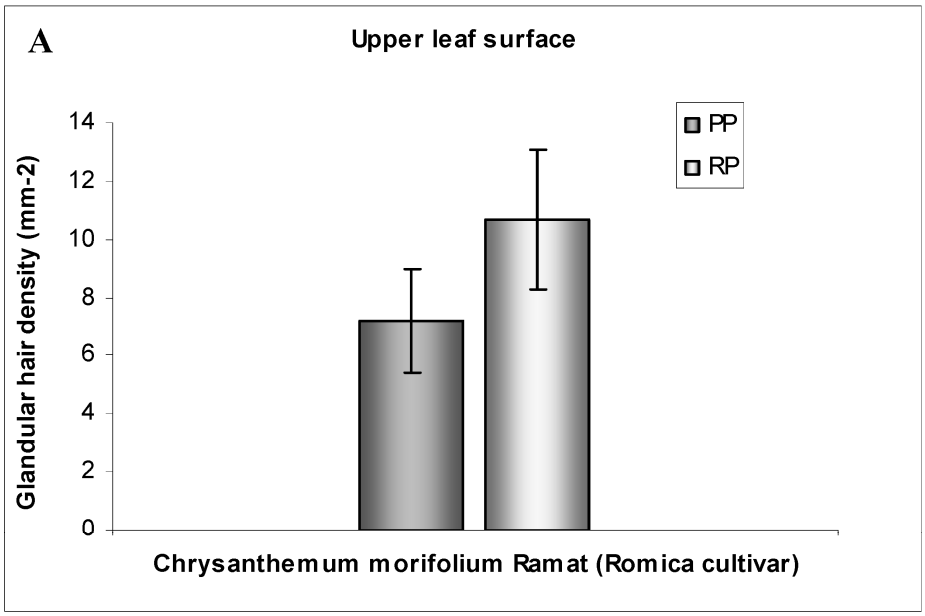


Fig. 2. Cross-section through the mesophyll of leaf in *Chrysanthemum morifolium* Ramat (Romica cultivar) parent plant (**a**) and *in vitro* regenerated plant (**b**).



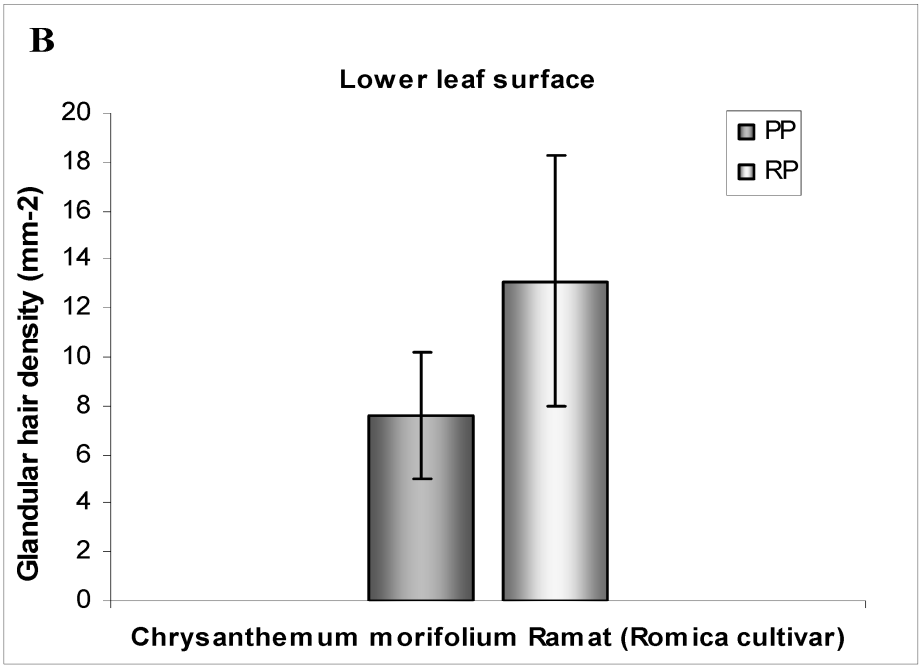


Fig. 3. Comparative glandular hair density on the upper (A) and lower (B) leaf surfaces in *Chrysanthemum morifolium* Ramat (Romica cultivar) parent plant (PP) and *in vitro* regenerated (RP) plant (±s.d., n=10).

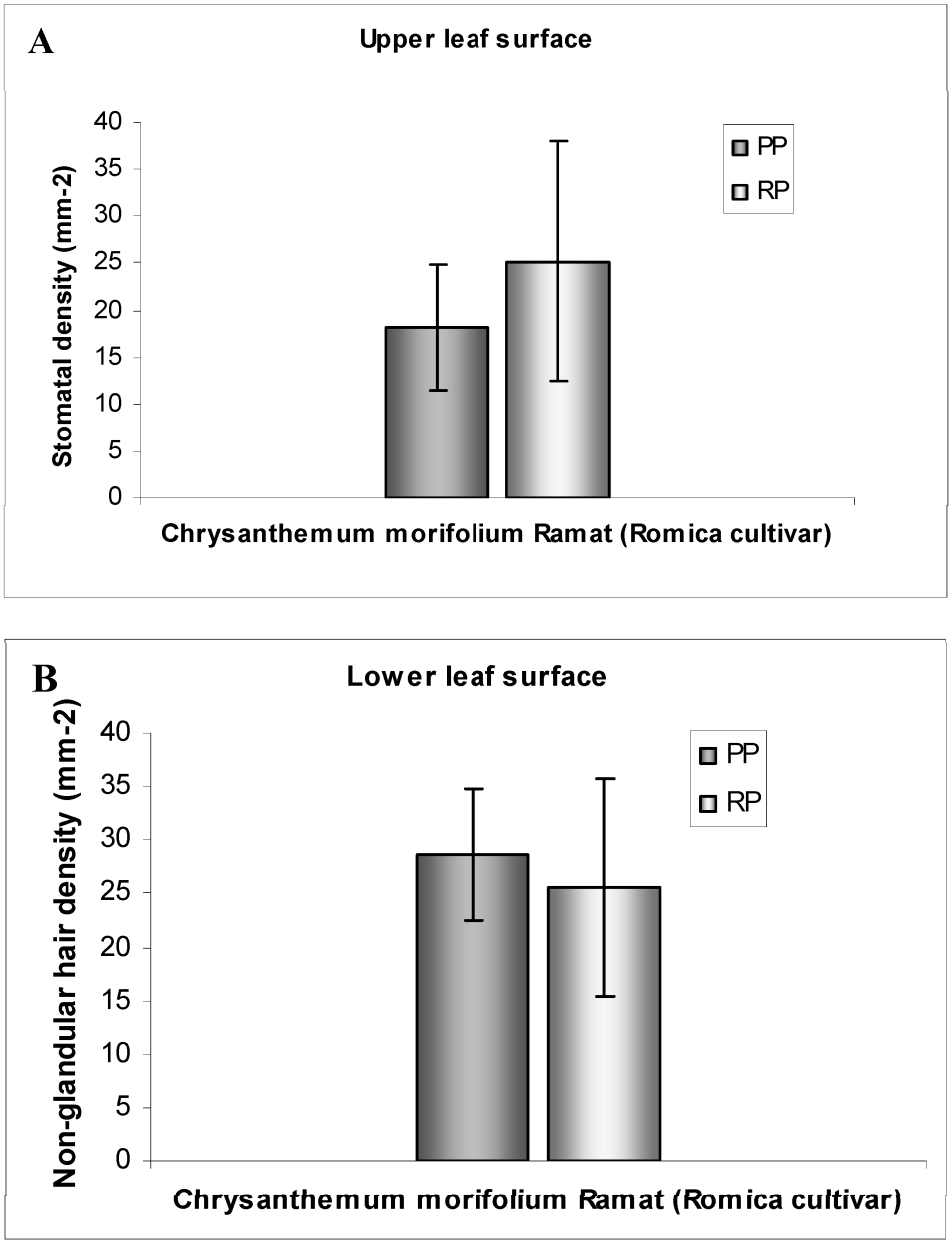


Fig. 4. Comparative non-glandular hair density on the upper (A) and lower (B) leaf surfaces in *Chrysanthemum morifolium* Ramat (Romica cultivar) parent plant (PP) and *in vitro* regenerated plant (RP) (±s.d., n=10).

REFERENCES

Bandyopadhyay T., Gangopadhyay G., Poddar R., Mukherjee K. K. (2004). Trichomes: their diversity, distribution and density in acclimatization of teak (*Tectona grandis* L.) plants grown *in vitro.* Plant Cell, Tissue and Organ Culture, 78: 113–121

Brutti C. B., Rubio E. J., Liorente B. E., Apostolo N. M. (2002). Artichoke leaf morphology and surface features in different micropropagation stage. Biol. Plant., 45: 197-204

Ducos, J.P., Alenton, R., Reano, J.F., Kanchanomai, C., Deshayes1, A. Petiard, V. (2003). Agronomic performance of *Coffea canephora* P. trees derived from large-scale somatic embryo production in liquid medium. Euphytica, 131: 215–223.

Gaspar T., Franck T., Bisbis B., Kevers C., Jouve L., Hausman J. F., Dommes J. (2002). Concepts in plant stress physiology. Application to plant tissue cultures. Plant Growth Regulation, 37: 263–285

Gonçalves, J. C.; Diogo, G. & Amâncio, S. (1998). *In vitro* propagation of chestnut (*Castanea sativa* x *C. crenata*): effect of rooting treatments on plant survival, peroxidase activity and anatomical changes during adventitious root formation. Scientia Horticult., 72:265-275.

Grout, B. W. W. & Aston, M. J. (1977). Transplanting of cauliflower plants regenerated from meristem culture. I. Water loss and water transfer related to changes in leaf wax and to xylem regeneration. Hort. Res., 17: 1-7.

Hazarika B.N. (2006). Morpho-physiological disorders in *in vitro* culture of plants. Scientia Horticulturae. 108 (2): 105-120.

Kishimoto S., Ohimiya A. (2006). Regulation of carotenoid biosynthesis in petals and leaves of chrysanthemum (*Chrysanthemum morifolium*). Physiologia Plantarum, 128 (3): 436-447.

Toma C., Cătuneanu D., Vidraşcu P., Toniuc A. (1985). Date de ordin histo-anatomic referitoare la unele soiuri de crizanteme (*Chrysanthemum morifolium* Ramat). An şt. Univ. „Al. I. Cuza" Iaşi, s. II a (Biol.), 31: 45-48

Ukiya M., Akihisa T., Tokuda H., Suzuki H., Mukainaka T., Ichiishi E., Yasukawa K., Kasahara Y., Nishino H. (2002). Constituents of *Compositae* plants. III. Anti-tumor promoting effects and cytotoxic activity against human cancer cell lines of triterpene diols and triols from edible *Chrysanthemum* flowers. Cancer Lett. 177 (1): 7-12.

Ukiya M., Akihisa T., Yasukawa K., Kasahara Y., Kimura Y., Koike K., Nikaido T., Takido M. (2001). Constituents of *Compositae* plants. 2. Triterpene diols, triols, and their 3-o-fatty acid esters from edible *Chrysanthemum* flower extract and their anti-inflammatory effects. J Agric Food Chem. 49 (7): 3187-97.

Vântu S. (2006). Organogenesis in *Chrysanthemum morifolium Ramat* (cultivar "*Romica*") callus cultures. An. şt. Univ. „Al. I. Cuza" Iaşi, s. II a (Biol. veget.), 51: 71-77

Vântu S. (2005). In vitro multiplication of *Chrysanthemum morifolium* Ramat. An. şt. Univ. „Al. I. Cuza" Iaşi, s. II a (Biol. veget.), 51: 75-81

Wang H. K., Xia Y., Yang Z. Y., Natschke S. L., Lee K. H. (1998). Recent advances in the discovery and development of flavonoids and their analogues as antitumor and anti-HIV agents. Adv. Exp. Med. Biol., 439: 191-225.

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