



## Structural responses of the photosynthetic apparatus of *Orthosiphon stamineus* Benth. to temperature stress after cryopreservation

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**ABSTRACT:** Histological structure of leaves from *in vitro* propagated *Orthosiphon stamineus* Benth. plants was investigated as follows: control plants without cryopreservation, plants, passed through cryopreservation, pre-treated in two ways (16 h sucrose 0.3M and 10 days ABA/abscisic acid/0.076 $\mu$ M) and adapted *ex vitro* micropropagated plants. Light microscopy analysis (LM) revealed that low temperature cause collapse of the photosynthetic tissues. Pre-treatment with sucrose diminishes stress effect. Scanning electron microscopy (SEM) analysis gave additional information about the structure of the epidermis of *in vitro* cultivated plants.

**KEY WORDS:** cryopreservation, structure, tissues, cuticle, *Orthosiphon stamineus*

Received 07 September 2009

Revision accepted 01 December 2009

UDK 582.929.4.085

### INTRODUCTION

*Orthosiphon stamineus* Benth. or Misai Kucing (Malay for "Cat's Whiskers"), *Lamiaceae*, is one of the most popular and widely used medicinal plant in South East Asia due to its biologically active compounds. Phenol content and total extract pharmacological activity point to the need for developing methods for long-term conservation of vital cell-lines with valuable medicinal characteristics for further analysis. Cryopreservation is an appropriate strategy for long-term conservation of plant genetic resources and plant tissues, possessing specific characteristics (GORDON-KAMM *et al.* 1990, ELLEUCH *et al.* 1998). A number of cryoprotective techniques are successfully applied to different medicinal plants in *in vitro* cultures (BAJAJ1995; ENGELMANN 1997). Meristematic cells in cryopreserved shoot-tips show less structural changes in comparison with the differentiated cells (VOLK & CASPERSEN 2007). The structure of the cells and tissues in differentiated leaves of plants, cultivated from successfully cryoprotected cells, is insufficiently investigated.

The aim of this study is to establish the structural organization of the photosynthetic apparatus on tissue level of *O. stamineus* plants, propagated *in vitro* without cryopreservation and plants regenerated after cryopreservation and to analyze the registered structural changes of fully developed leaves, which may become representative of probable mechanisms of overcoming the low temperature stress.

### MATERIALS AND METHODS

**Plant material.** Tree variants from *O. stamineus in vitro* cultivated plants are studied: control plants (*in vitro* propagated plants without cryopreservation), plants, undergone cryopreservation by vitrification (URBANOVÁ *et al.* 2006) pre-treated by two methods (16 h sucrose 0.3M and 10 days ABA/abscisic acid/0.076 $\mu$ M) and adapted *ex vitro in vitro* propagated plants.

**Methods.** LIGHT MICROSCOPY (LM). Leaf material was fixed in 3% glutaraldehyde (pH 7.4) and used for light

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microscopy studies. Cross sections were cut by hand. Light microscopy study was led on Amplival 4 microscope (Carl Zeiss, Jena, Germany). Morphometric data are average of 15 measurements for each histological parameter: thickness of the leaf lamina, assimilative parenchyma, and epidermis.

SCANNING ELECTRON MICROSCOPY (SEM). For SEM parts of fixed leaf material were used. The leaf tissue was dehydrated with ethanol in ascending concentrations. The samples were covered with 0.1 nm golden layer in vacuum-evaporator Jeol JFC-1200 fine colter and observed by means of scanning electron microscope Jeol JSM-5510.

## RESULTS AND DISCUSSION

The leaf of *O. stamineus* is dorsi-ventral with one layer palisade tissue, hypostomatic. Adaxial and abaxial epidermis are similarly structured with non-glandular and glandular trichomes. This is the way in which were organized leaves of *ex vitro* grown plants (Fig 1). In this study, these plants, which are product of successful adaptation after micropropagation, were experimental equivalent of the native species. The leaf lamina average thickness was  $39.60 \pm 2.48 \mu\text{m}$  (Tab. 1) where the part of assimilative parenchyma was about 71%. The proportion between autotrophic and heterotrophic tissues was 2:1. *In vitro* propagated plants, as a control equivalent of the experiment, had the same histological organization of the photosynthetic apparatus (Fig. 2). Evident, however, were the differences in morphometric data for the two examined variants. The average thickness of the leaf lamina of *in vitro* cultivated plants was about 47% smaller than this of *ex vitro* grown plants. Analysis revealed that approximately 50% reduction of the thickness referred to all examined tissues (Tab. 1) and there were not considerable changes in palisade factor values.

The morphological characteristic of the leaves from *in vitro* cultured plants was complemented by the SEM

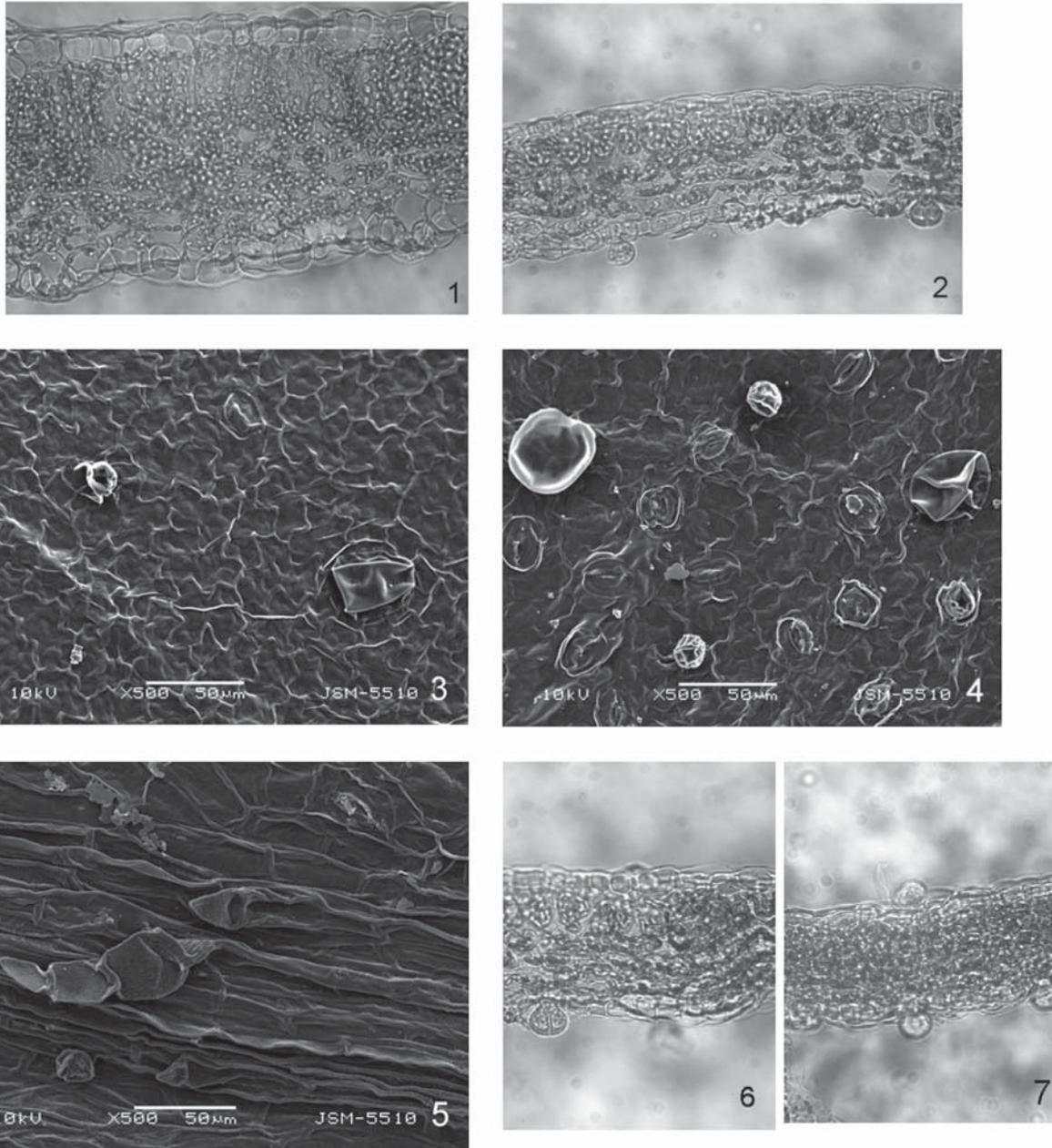
analysis. The cuticle of the adaxial and abaxial leaf surfaces was thin and fine striated. The epidermal cells were polygonal with undulate anticlinal walls (Figs. 3, 4). Non-glandular and two types of glandular trichomes – peltate and capitate were distributed on both leaf sides. The peltate trichomes had very short one-celled stalks and large four-celled heads and they were partially sunken in the epidermis (Fig. 4). The capitate trichomes consisted of basal cell, a short stalk cell and a bicellular head (Fig. 3). The non-glandular trichomes were uniseriate pointed consisting of one to four cells with warty cell walls and they were confined to the major veins (Fig. 5). The type of the stomata was diacytic (Fig. 4). However, in recent morphological study of the two varieties of *O. stamineus* grown in Malaysia (KENG & SIONG 2006) the authors did not report the capitate type of trichomes. The types of trichomes and stomata were representative of the *Lamiaceae* species (METCALFE & CHALK 1965). The same type of glandular trichomes were found also in species of the genera *Ocimum* (WERKER *et al.* 1993), *Salvia* (SERRATO-VALENTI *et al.* 1997; BISIO *et al.* 1999; ÖZKAN 2008), *Thymus* (SATIL *et al.* 2005; MARIN *et al.* 2008) and *Teucrium* (PAROLLY & ÖZKAN 2007).

Plants regenerated after cryopreservation were characterized with smaller thickness of the leaf lamina as well as the inner tissues. The morphometric deviations between leaves of control plants and those of variant pre-treated with sucrose during cryopreservation were non-essential. The thickness of the leaf lamina in cryopreserved plants was smaller only by 6% which is due to decrease of the palisade parenchyma thickness. The light microscopy observation revealed collapsed palisade cells, whose shape is not typical and resembled to the spongy cells' one (Fig. 6). This slightly diminished the palisade factor (43.45%).

In general the tendency of decreasing tissues' thickness was manifested in plants pre-treated with ABA. The leaf lamina was around 30% thinner. Noticeable change of mesophyll architecture was established (Fig. 7). Its

**Table 1.** Thickness [ $\mu\text{m}$ ] of leaf lamina and its tissues and palisade factor [%] in *in vitro* cultured *O. stamineus* without and after cryopreservation and *ex vitro* plants.

Variants	Lamina	Mesophyll	Palisade parenchyma	Spongy parenchyma	Adaxial epidermis	Abaxial epidermis	Palisade factor
<i>Ex vitro</i>	$39.60 \pm 2.48$	$28.16 \pm 1.81$	$12.56 \pm 1.00$	$15.68 \pm 1.24$	$5.28 \pm 0.76$	$5.12 \pm 0.71$	44.47%
Control plants	$20.80 \pm 2.75$	$15.04 \pm 3.23$	$6.72 \pm 1.56$	$7.84 \pm 1.63$	$2.24 \pm 0.42$	$3.04 \pm 0.62$	46.15%
16 h 0.3M sucrose	$19.60 \pm 1.91$	$13.68 \pm 1.86$	$5.84 \pm 0.89$	$7.60 \pm 1.55$	$2.32 \pm 0.31$	$3.60 \pm 0.79$	43.45%
10d 0.076 $\mu\text{M}$ ABA	$14.50 \pm 3.33$	$9.76 \pm 2.26$	$4.48 \pm 1.06$	$5.12 \pm 1.24$	$2.00 \pm 0.59$	$2.48 \pm 0.71$	46.66%



**Fig. 1.** Anatomical structure of leaf in *ex vitro* plants (LM) (x 80).

**Fig. 3.** *Orthosiphon stamineus* - adaxial epidermis – epidermal cells and trichomes.

**Fig. 5.** *Orthosiphon stamineus* - abaxial epidermis – non-glandular trichomes.

**Fig. 2.** Anatomical structure of *in vitro* cultivated control plants (LM) (x 80).

**Fig. 4.** *Orthosiphon stamineus* - abaxial epidermis – epidermal cells, trichomes and stomata.

**Fig. 6 and 7.** Anatomical structure of leaves in *in vitro* cultured plants after cryopreservation pre-treated with sucrose(6) and with ABA (7) (x 80).

thickness was 35% lower due to the cell collapse of the whole autotrophic tissue. The apoplast volume was highly reduced and palisade and spongy parenchyma were hardly distinguishable. The assimilative parenchyma was morphologically homogeneous structured. Cultivation itself might disturb the forming of anatomical structures (MEDVEDEVA 2008), but in these experimental conditions low temperature was a decisive factor. Collapsed mesophyll cells were universal reaction to low temperatures in experimental as well as natural conditions (REINIKAINEN & HUTTUNEN 1989, BÄCK *et al.* 1993). On the other hand the increasing of the simplast surface between photosynthetic cells was very important for normalization of the cell metabolism and transport of assimilates in low temperature stress conditions. The role of saccharides in overcoming the stress has been widely discussed in literature (PYANKOV & VASKOVSKII 1994; PSHYBITKO *et al.* 2003). In our experimental conditions we might assert, that for cryopreserved plants pre-treated with ABA low temperature stress was evident and histogenesis lead to optimal configuration of the autotrophic tissue. Our statement was based on morphometric indices – despite decreased thickness of the palisade tissue by around 60% in comparison with *ex vitro* grown plants, the palisade factor was even slightly higher (46.15%). Probably this was the most actively functioning structure in the experimental conditions for this variant. Concerning cryopreserved plants pre-treated with sucrose no significant morphometric differences from the control variant were observed which could be explained by preventive role of sucrose.

## CONCLUSION

Results showed that even on tissue level sucrose may decrease stress effect since it is known that carbohydrates increase photosynthetic cells' stability in sub-cellular level (SOPINA *et al.* 1994). The structural investigation suggests that low temperatures harm the assimilative parenchyma, but there are compensative overcoming mechanisms, while epidermal tissues are much more stable and significant changes are not observed.

**Acknowledgements** – This work was funded by a grant of Sofia University “St. Kl. Ohridski” – SU – 094/2009 and a grant of Bulgarian Ministry of Education – BG/SK-101/2007.

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**REZIME**

## Strukturni odgovor fotosintetskog aparata *Orthosiphon stamineus* Benth. na temperaturni stres posle krioprezervacije

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**H**istološka struktura listova biljaka *Orthosiphon stamineus* Benth. propagiranih u *in vitro* uslovima je istraživana na nekoliko grupa biljaka: kontrolne biljke bez krioprezervacije, biljke koje su prošle kroz postupak krioprezervacije prethodno tretirane na dva načina: 16 h na 0.3M saharozu i 10 dana na 0.076 $\mu$ M apscisinskoj kiselini, a yatum su adaptirane na *ex vitro* uslove gajenja. Istraživanje na svetlosnom mikroskopu već pokazuje da niska temperatura izaziva kolaps fotosintetičkih tkiva. Prethodni tretman sa saharozom umanjuje stresni efekat. Analiza skening-elektronskih mikrofografija daje dodatne informacije o promenama na epiderimsu *in vitro* gajenih biljaka.

**Ključne reči:** krioprezervacija, struktura, tkiva, kutikula, *Orthosiphon stamineus*