In vitro and *in vivo* variability of histological traits of *Dianthus callizonus* (*Caryophyllaceae*) aerial vegetative organs

Anca Păunescu

Institute of Biology, Romanian Academy, 296 Splaiul Independenței St., 060031 Bucharest, Romania, e-mail: ancuta_paun@yahoo.com

Received: October 09, 2008 ▹ Accepted: November 10, 2008

Abstract. Although *in vitro* culture techniques offer reliable tools for *ex situ* conservation of the endangered plants, variability may occur as a consequence of tissue culture specific conditions. A comparative study of the histological traits of in *vitro* and *in vivo* grown *Dianthus callizonus* plants has revealed numerous anatomical differences, such as reduced deposition of epicuticular waxes, blocked and abnormally positioned stomatal apparatus, abnormally shaped cell epidermis, altered trichome morphology, hypertrophy of the mesophyll and stem cortex, hypolignification, and absence of calcium oxalate crystals. Anatomical variability of the *in vitro*-grown plants was interpreted as an adaptive response to *in vitro* culture conditions.

Key words: *Dianthus callizonus*, histological variability, *in vitro* culture

Introduction

Feasibility of using *in vitro* culture techniques for the endangered plant genetic resources conservation was recognized late in the 1970s (Sarasan & al. 2006). Presently, *in vitro* methods have found increasing significance as complementary means to *in situ* plant conservation, especially for vegetatively propagated and recalcitrant seed-producing species (Withers & Engelmann 1997). Although the development of successful *in vitro* propagation and storage protocols are reliable methods that make possible the establishment of extensive germplasm collections, the specific culture conditions can cause changes in plants and lead to progenies with modified characteristics, both heritable (genetic) or non-heritable (epigenetic) (Jain 2001). Variability has been documented to occur at physiological, morpho-anatomical, karyotypic, and biochemical levels (Apostolo & Lorente 2000; Kitin & al. 2005; Sarasan & al. 2006; Zhao & al. 2006). Maintaining the genetic identity of the donor plant and a completely viable acclimatised plant yield after *in vitro* storage are the essential prerequisites for using *in vitro* culture techniques for *ex situ* conservation purposes.

Although many altered characteristics of the *in vitro* regenerated plants have been reported (Hempel 1989), the state of knowledge of plant tissue reactions to artificial *in vitro* conditions is still insufficient; therefore more researches should address this area (Withers & Engelmann 1997; Sarasan & al. 2006). The integrity and stability of *in vitro* grown plants is monitored by means of morpho-anatomical, biochemical and molecular parameters, or markers. The first assessment stage includes phenotype analysis through comparative morphological and histological studies.

Dianthus callizonus Schott & Kotschy is a stenoendemic plant taxon in the Romanian flora, maintained in the *in vitro* plant germplasm collection of the Institute of Biology (Bucharest, Romania).

This is the first report of the anatomical status of *D. callizonus* preserved germplasm. The procedure described should serve as a useful tool in estimating the efficiency of the conservation method and for optimizing the acclimatization stage.

Material and methods

Plant material

The plant material was collected in late July 2007, from populations in a flowering stage, naturally growing in the Piatra Craiului National Park, Brasov District, Romania. In order to compare the histological traits, regenerative tissue from *in vitro* maintained collection was grown for an entire cycle, according to an earlier reported protocol (Paunescu & Holobiuc 2003).

Histological studies

For the histological investigation, fresh tissue samples were fixed for two weeks in a formalin buffer solution (15 % v/v formalin, 2 % w/v sodium bicarbonate). Dehydration was conducted at room temperature in graded ethanol series (5 to 100 %), followed by clearing with xylene and embedding in paraffin, according to the usual procedures (Dashek 2000). Serial sections (7-10 μm thick) were cut with a rotary microtome and transferred onto glass slides. The paraffin was removed and the sections were rehydrated and double stained with 1 % w/v night green and carmine alum (2 % w/v carmine, 5 % w/v aluminum potassium sulfate), and finally mounted in synthetic resin (Entallan). For surface view of the epidermis, leaf peelings were colored with 1 % w/v safranin. Histological observations were made both with bright and dark-field microscopy, under a Nikon Eclipse E200 microscope, and micrographs were recorded with a Nikon Coolpix 5400 digital camera.

Results

In general, *in vitro* developed plants had smaller leaves and shorter and thinner stems.

Leaves

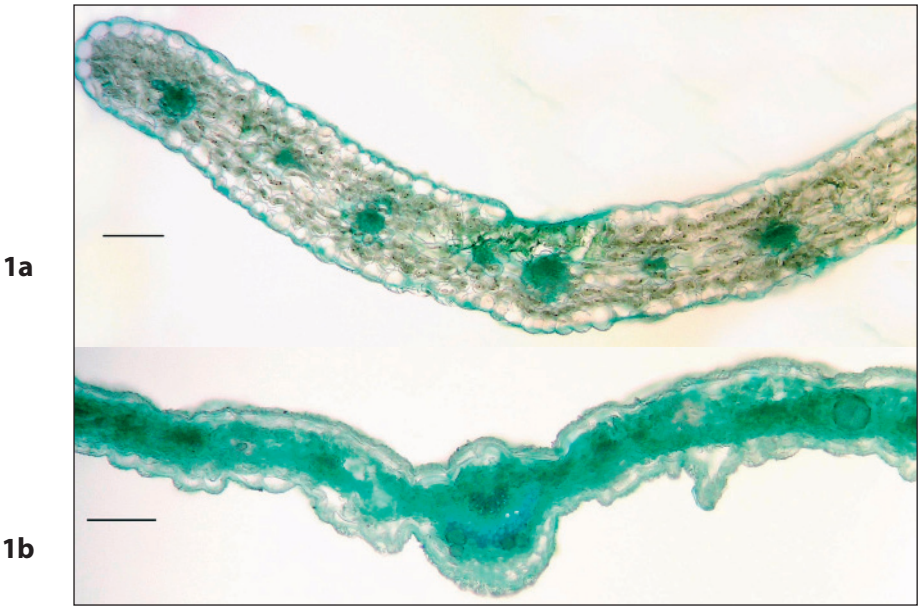
The structure of *in vitro* developed leaves differed in certain features from normal *in vivo* naturally grown leaves. The differences concerned the general leaf shape in cross-section, cuticular wax development, trichome density and morphology, epidermal cell wall shape, disposal and function of the stomata, and calcium oxalate crystals formation. Lamina cross-sections of *in vitro* developed leaves was flat, displaying medium-sized epidermal cells, with reduced deposition of epicuticular waxes (Plate I, Fig. 1a) as compared with the naturally grown which show a prominent midrib, mostly on the abaxial surface, and very large-sized epidermal cells, with the external walls cutinized and covered by a thick and striated epicuticular wax layer (Plate I, Fig. 1b). Uniseriate trichomes, occurring exclusively on the edge of the leaf, were less frequent, shorter and with a rounded apex (Plate I, Fig. 2a) as compared with the dense, long and acuminated naturally grown hairs (Plate I, Fig. 2b). In surface view, the epidermal cell walls were deeply sinuated and the stomatal apparatus, mostly of anomocytic type, appeared with a blocked wide open pore (Plate II, Fig. 1a), contrary to the normal leaf with straight, or slightly undulated epidermal cell walls and diacytic type stomata, showing a functional closed pore (Plate II, Fig.1b).

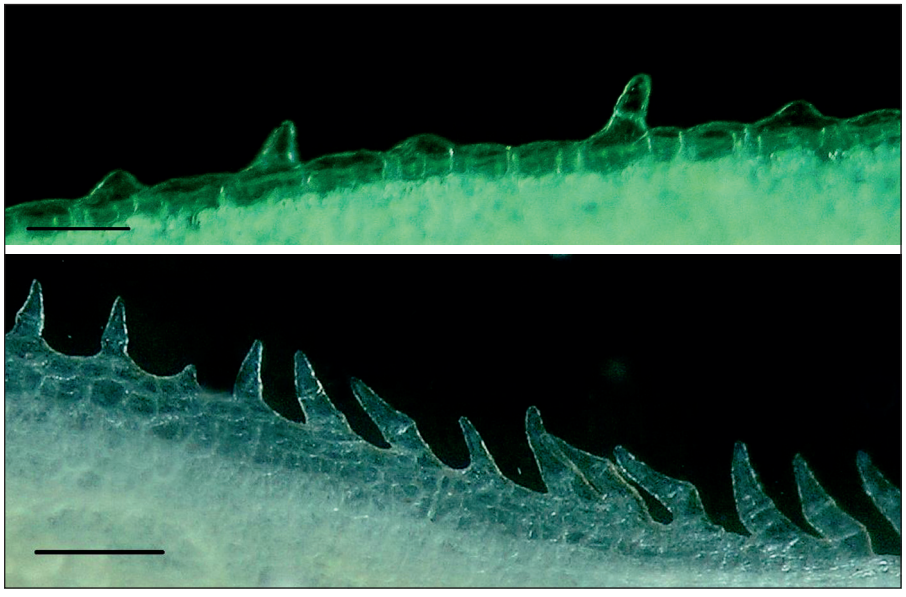
Dark-field images of the entire leaf revealed the absence of calcium oxalate crystals in micropropagated leaves (Plate II, Fig. 2a), while those naturally grown displayed abundant conspicuous cluster-crystals (Plate II, Fig. 2b).

Stem

Anatomical analysis of the micropropagated stem revealed some abnormalities which include stomata position, cortex and pericyclic sclerenchyma development, medullary parenchyma distribution, and calcium oxalate crystals yield. In transverse section stomata appeared raised, as compared with the epidermal cell layer (Plate III, Fig. 2a), cortical parenchyma was hypertrophied (5-6 layers of non-photosynthetic large-sized cells, with thin walls and large intercellular spaces), pericyclic sclerenchyma was poorly developed (discontinuous ring of one-cell layer), medullary parenchyma was persistent, and calcium oxalate crystals were absent (Plate III, Fig. 1a). In contrast, normal stems showed a lower position of the stomata (Plate III, Fig. 2b), a thinner cortical parenchyma made of 3-4 layers of medium-sized cells (the outer two layers were photosynthetic), with small intercellular spaces, pericycle characterized by a broad sclerenchymatous ring (5-6 layered), disorganised medullary parenchyma enclosing a central pith cavity, and frequent crystals formation in the proximity of the pericyclic sclerenchymatous ring (Plate III, Fig. 1b).

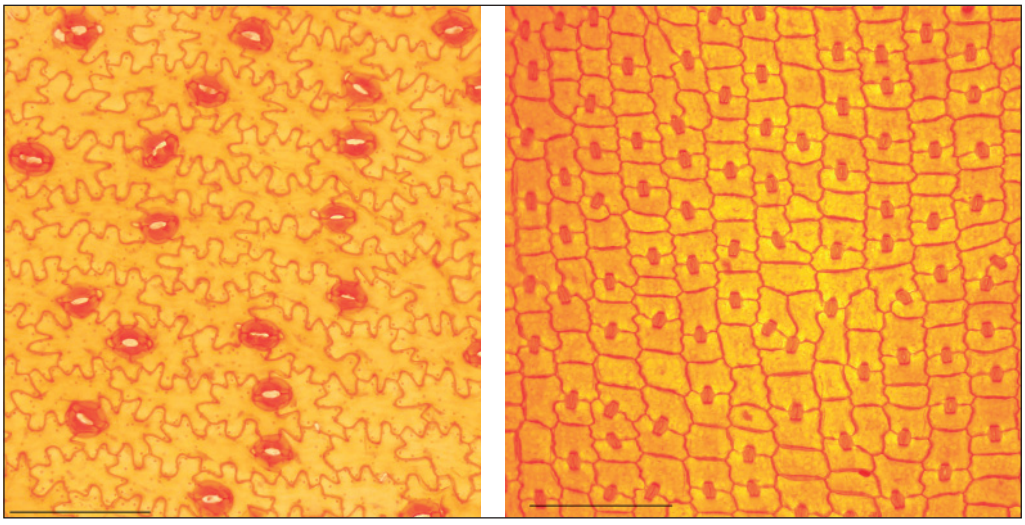
Plate I



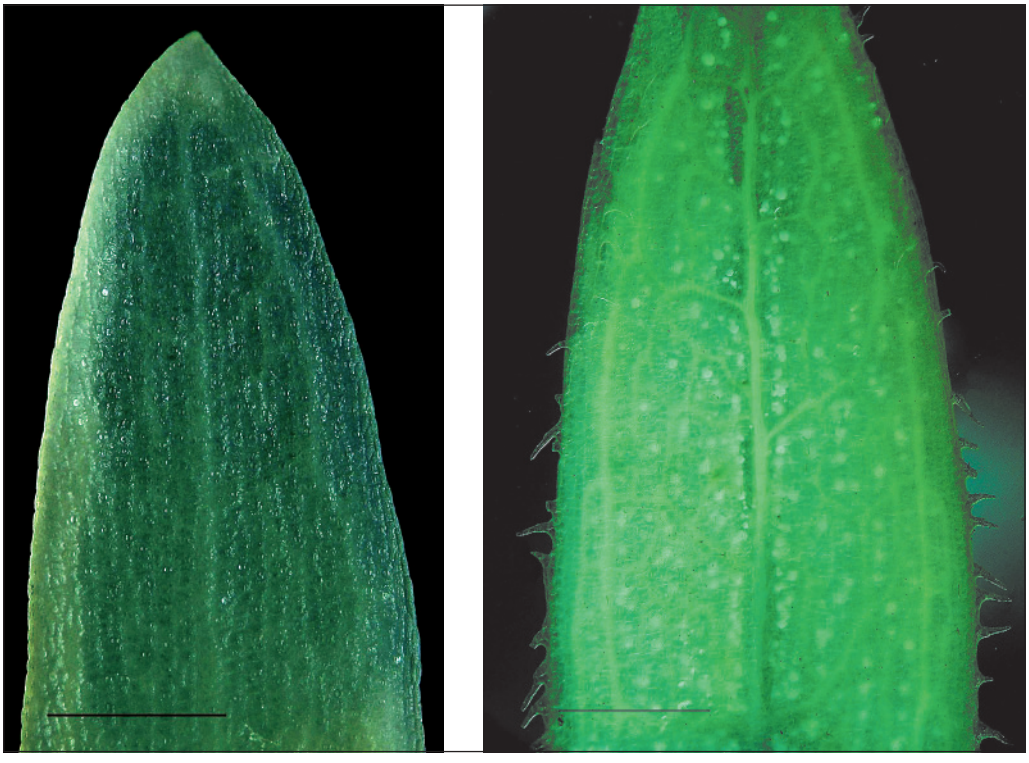


Figs 1–2. Foliar changes in anatomical characteristics: **a** – *in vitro* regenerated plant; **b** – wild plant; **1,** Leaf cross-sections. Scale bar = 500 µm; **2,** Leaf trichomes. Scale bar = 200 µm.

Plate II



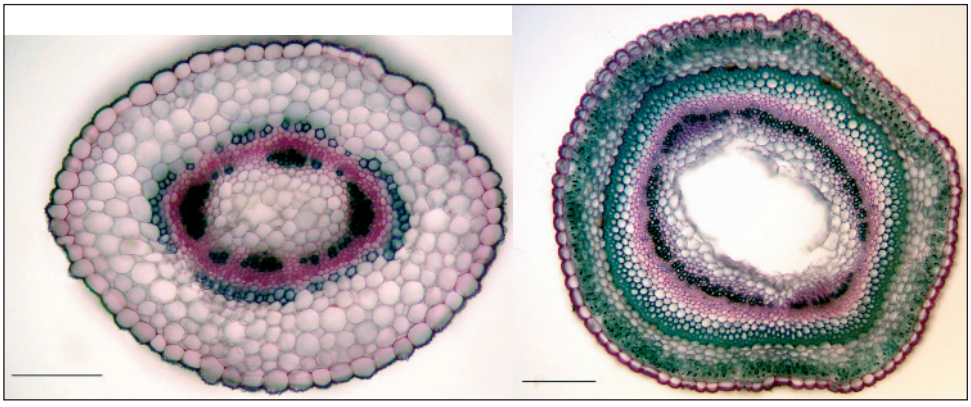
1a 1b



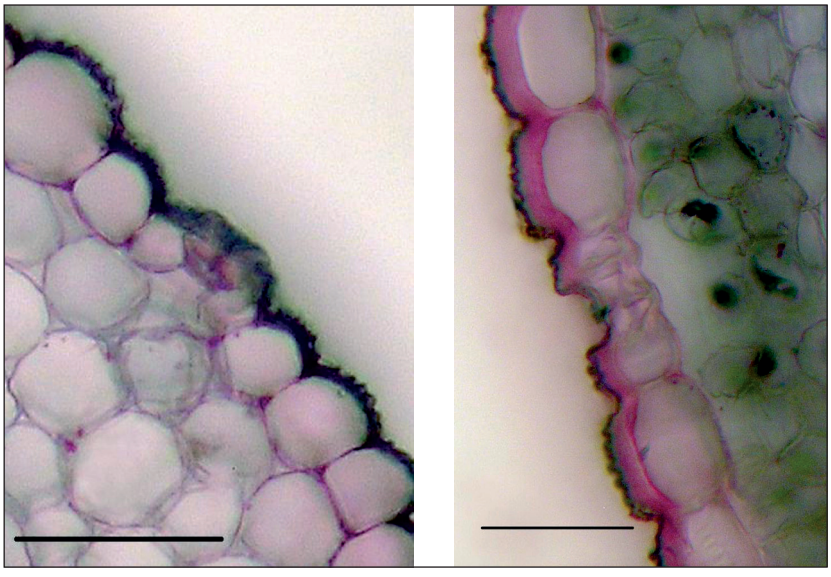
2a 2b

Figs 1–2. Foliar changes in anatomical characteristics: **a** – *in vitro* regenerated plant; **b** – wild plant; **1,** Epidermis surface view. Scale bar = 200 µm; **2,** Leaf view. Scale bar = 1 mm.

Plate III



1a 1b



2a 2b

Figs 1–2. Stem changes in anatomical characteristics: **a** – *in vitro* regenerated plant; **b** – wild plant; **1,** Stem cross-sections. Scale bar = 200 μm; **2,** Stomata position. Scale bar = 50 μm.

Discussion

It is well known that most histological traits of the plant are under the control of environmental conditions. Organs of an individual plant living under stress undergo adaptive changes which determine their more effective function (Bitonti & al. 1996). *In vitro* cultured plants are exposed to specific artificial conditions which differ from those in the natural environment. These specific conditions are responsible for the structural changes occurring in micropropagated plants. The main determinant factors are high relative air humidity, air composition and culture media content.

The air humidity in culture vessels is very high, ranging from 95 % to 100 %. The plant response is to enhance water diffusion into the cells, with subsequent increase in both parenchymatic cells size and intercellular spaces. An immediate consequence is hypertrophy of the parenchymatic cells (mesophyll, cortex and pith). Furthermore, the continuing cell enlargement retards the development of secondary walls (lignification and cutinization) (Taji & al. 1996) resulting in thinner cell walls, reduced deposition of epicuticular waxes, and poor mechanical tissue formation. Water saturated flask atmosphere and the accumulation of specific gases (ethylene) in the confined atmosphere increase dramatically the transpiration rate and finally determine the failure of stomata to close (Kevers & al. 2004). The risk of stomata malfunctioning is increased by altered (reduced and disoriented) cellulose biosynthesis in the guard cells wall (Ziv 1991) as a consequence of culture media growth regulators content (Paek & Hahn 2000).

The observed leaf and stem structural abnormalities did not result from the occurrence of somaclonal variation phenomena but are a response to stress caused by the *in vitro* culture conditions, which corresponds to the initial symptoms of hyperhydricity described by Kevers & al. (2004). When transferred from *in vitro* vessels to *ex vitro* conditions, the plants exhibited severe water loss and thus a low survival rate during the weaning period. Some authors (Ziv 1991; Kevers & al. 2004; Joshi & al. 2006) have suggested that the gradual exposure of plantlets to the external conditions for a sufficiently long period could raise considerably the survival rate. Furthermore, improvement of culture conditions like ethylene removal by a proper culture vessel ventilation (Majada & al. 2000), reduction or elimination of BAP (6-benzylaminopurine) and IAA (Indole-3-acetic acid), and increase of the concentration of iron and/or magnesium in culture media (Yadav & al. 2003) resulted in successful normal plant regeneration and efficient subsequent acclimatization.

Recently, Digiuni & al. (2008) have demonstrated experimentally that the trichome patterning system was robust and could yield a similar qualitative output, despite the naturally occurring perturbations in the form of genetic and environmental variability. In this sense, it was logical to assume that the rounded apical shape of *in vitro* developed trichomes was also a consequence of water saturated atmosphere in culture vessels. It was induced by the terminal cell turgescence, and not a result of a genetically altered trichome patterning.

The absence of calcium oxalate crystals in micropropagated plants seemed to be a consequence of culture media calcium content. *Dianthus callizonus* was described as a calcicolous plant, naturally grown on alkaline mineral soil with high calcium availability (Onete & al. 2006), ranging in soil solution from 80 to 145 meq/L (Paunescu unpubl.). In contrast, in culture media composition calcium was in low concentrations (3 meq/L). The absence of calcium oxalate crystals from micropropagated plants reported in the present study strongly confirmed the earlier reviewed findings by Franceschi & Nakata (2005). These studies lead to the hypothesis that calcium oxalate formation is the end result of a mechanism for controlling calcium excess at the tissue and organ levels. In addition, our findings confirmed the conclusions of Ayala-Cordero & al. (2006) about the positive correlation of the accumulation of calcium oxalate crystals and the stress induced by water deficit.

Understanding of the various factors involved in the control of morphogenesis in micropropagated plants and their proper management is prerequisite to optimize regeneration and maintenance protocols for an efficient *in vitro* conservation of plant germplasm.

Acknowledgements. The author is grateful to the anonymous reviewer for her/his critical comments.

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