Journal of Plant Physiology 171 (2014) 1344–1353

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Physiology

Adaptive changes in photosynthetic performance and secondary metabolites during white dead nettle micropropagation

V. Kapchina-Toteva[a], M.A. Dimitrova[a], M. Stefanova[b], D. Koleva[b], K. Kostov[c], Zh.P. Yordanova[a], D. Stefanov[d], M.K. Zhiponova[a,*]

[a] Department of Plant Physiology, Faculty of Biology, Sofia University "St. Kliment Ohridski", 8 Dragan Tsankov Blvd., 1164 Sofia, Bulgaria
[b] Department of Botany, Faculty of Biology, Sofia University "St. Kliment Ohridski", 8 Dragan Tsankov Blvd., 1164 Sofia, Bulgaria
[c] AgroBioInstitute, Buld. Dragan Tzankov 8, 1164 Sofia, Bulgaria
[d] Department of Biophysics, Faculty of Biology, Sofia University "St. Kliment Ohridski", 8 Dragan Tsankov Blvd., 1164 Sofia, Bulgaria

ARTICLE INFO

Article history:
Received 21 December 2013
Received in revised form 12 May 2014
Accepted 13 May 2014
Available online 16 June 2014

Keywords:
Medicinal plants
Micropropagation
Leaf
Photosynthesis
Secondary metabolites

ABSTRACT

The white dead nettle, *Lamium album* L., is an herb that has been successfully cultivated under *in vitro* conditions. The *L. album* micropropagation system offers a combination of factors (light intensity, temperature, carbon dioxide (CO_2) level, humidity) that are limiting for plant growth and bioactive capacity. To get a better understanding of the mechanism of plant acclimation towards environmental changes, we performed a comparative investigation on primary and secondary metabolism in fully expanded *L. album* leaves during the consecutive growth in *in situ*, *in vitro*, and *ex vitro* conditions. Although the genetic identity was not affected, structural and physiological deviations were observed, and the level of bioactive compounds was modified. During *in vitro* cultivation, the *L. album* leaves became thinner with unaffected overall leaf organization, but with a reduced number of palisade mesophyll layers. Structural deviation of the thylakoid membrane system was detected. In addition, the photosystem 2 (PS2) electron transport was retarded, and the plants were more vulnerable to light damage as indicated by the decreased photoprotection ability estimated by fluorescence parameters. The related CO_2 assimilation and transpiration rates were subsequently reduced, as were the content of essential oils and phenolics. Transfer of the plants *ex vitro* did not increase the number of palisade numbers, but the chloroplast structure and PS2 functionality were recovered. Strikingly, the rates of CO_2 assimilation and transpiration were increased compared to *in situ* control plants. While the phenolics content reached normal levels during *ex vitro* growth, the essential oils remained low. Overall, our study broadens the understanding about the nature of plant responses towards environmental conditions.

Introduction

The micropropagation technique used for rapid plant multiplication offers opportunities for basic research on plant growth and biosynthetic capacity (Dimitrova et al., 2010; Stefanova et al., 2011; Aremu et al., 2013). Notably, the cultivation under *in vitro* conditions could be accompanied by alterations in the genetic identity, which should be strictly followed to assure the preservation of the genetic identity characteristic for the species, which defines the related plant biosynthetic capacity (Zhang et al., 2010). One of the most common problems in micropropagation is the quantitative and qualitative variability in plant responses due to the environmental conditions (Ashihara et al., 2011; Aremu et al., 2013). During the *in vitro* cultivation, the plantlets grow under controlled nutritional and physical conditions with a mixotrophic and/or heterotrophic growth, depending on external sugar source

Abbreviations: CO_2, carbon dioxide; $HgCl_2$, mercury(II) chloride; MS, Murashige and Skoog medium; $KMnO_4$, potassium permanganate; Chl, chlorophyll; dNTP, deoxyribonucleotide triphosphate; DNA, deoxyribonucleic acid; E, excess light energy; F_m and F_m', maximal fluorescence level (for dark-adapted and light-adapted plants, respectively); F and F_0, fluorescence level (when applying actinic light and the dark level of fluorescence, respectively); F_v, variable fluorescence; Φ_{max}, maximal quantum yield of PS2; Φ_{PS2}, effective quantum yield; ISSR, inter-simple sequence repeat; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; NPQ, non-photochemical fluorescence quenching; PCR, polymerase chain reaction; PPFD, photosynthetic photon flux density; PS1 and 2, photosystem 1 and 2; qP, photochemical fluorescence quenching coefficient; RAPD, random amplified polymorphic DNA; RI, retention index; ROS, reactive oxygen species.

* Corresponding author. Tel.: +359 886680589; fax: +359 2 8656641.
E-mail address: miroslava_zhiponova@abv.bg (M.K. Zhiponova).

http://dx.doi.org/10.1016/j.jplph.2014.05.010
0176-1617/

(Le et al., 2001). Micropropagated plants are developed in the presence of higher humidity, lower irradiance, limited air turbulence, and the plantlets are subjected to increased risk of bacterial and fungal infections (Pospíšilová et al., 1999a). Due to the *in vitro* cultivation, the inflow of carbon dioxide (CO_2) and outflow of gaseous plant products decrease (Pospíšilová et al., 1999a,b). The leaves of *in vitro*-grown plants showed deviations in chloroplast structure, as well as lower photosynthetic activity, and reduced metabolic activity (Hazarika, 2003). These effects were correlated with not completely active photosynthetic apparatus as estimated by photosystem 2 (PS2) fluorescence parameters (Christov et al., 2007). In such case, the efficiency of micropropagation techniques is restricted, especially when plantlets were transferred to *ex vitro* greenhouse or field conditions (Pospíšilová et al., 1999a).

The most expressed and rapidly induced changes after transfer to the field occur in leaf anatomy and the photosynthetic processes (Wetzstein and Sommer, 1982; Hazarika, 2003). Short-term acclimation studies have revealed a good development of the photosynthetic apparatus estimated by fluorescence parameters, but a decrease was established during leaf aging (Cassana et al., 2010). Improving the function of photosynthesis during acclimation to *ex vitro* conditions is essential to assure high survival rates and metabolic performance after transplantation to field conditions (Pospíšilová et al., 1999a,b). It was demonstrated that CO_2 enrichment can improve acclimation of *in vitro*-grown plantlets to *ex vitro* conditions (Pospíšilová et al., 1999b). Interestingly, it has been shown that, after *in vitro* growth followed by long-term *ex vitro* acclimation, the plants showed better field survival and increased growth rates (Talavera et al., 2005).

The variable environmental conditions affect the primary metabolism and, in aromatic plants, trigger respective modulation in the content of secondary metabolites as well. Bioactive compounds as essential oils and phenolic compounds play an important role in plant environmental interactions including both biotic and abiotic factors. The essential oils are released as fragrances from the plant to attract pollinators or deter herbivores (Ashihara et al., 2011). The phenolic compounds are characterized by antioxidative activity that prevents the accumulation of cell-destructive reactive oxygen species (ROS) produced in metabolic reactions (Bonoli et al., 2004). Under stress conditions such as drought, the photosynthesis is inhibited by stomata closure, which limits CO_2 uptake from the air. As a result, while light is absorbed and utilized for the generation of reduction equivalents, such as $NADPH + H^+$, the subsequent use of these reduced molecules for CO_2 fixation has declined. The resulting over-reduction has been associated with generation of ROS (Selmar and Kleinwächter, 2013). As a consequence, the metabolic processes are shifted towards biosynthetic activities that consume reduction equivalents. Accordingly, an enhanced synthesis of reduced compounds, such as isoprenoids, phenols or alkaloids, has been observed.

The white dead nettle, *Lamium album* L., is a perennial herb from the genus *Lamium* (family *Lamiaceae*) known for a wide spectrum of therapeutic activities defined by its enriched phytochemical content (Alipieva et al., 2003; Matkowski and Piotrowska, 2006; Yordanova et al., 2014). *L. album* micropropagation requires low energy inputs and offers an experimental system to study the effect of growth conditions on plant growth and biosynthetic capacity (Dimitrova et al., 2010; Valyova et al., 2011). In the current study, we assessed the effects of environmental conditions (during *in situ*, *in vitro*, and *ex vitro* growth) on structural and functional photosynthetic parameters of *L. album* leaves, and we examined correlations with the subsequent modulation in the content of essential oils and phenolics.

Materials and methods

Plant material and in vitro cultivation

Lamium album L. plants were grown *in situ* in their natural habitat in the Lozen Mountain, Sofia, Bulgaria (Supplementary Fig. S1a). The voucher specimen SO105183 was deposited in the Herbarium of Sofia University "St. Kliment Ohridski". Fully expanded leaves taken from the middle part of the stem were collected in the period of blooming (in May).

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2014.05.010.

For *in vitro* cultivation, mono-nodal 1–2 cm stem segments from the *in situ*-grown plants were thoroughly washed with tap water and sterilized in 0.1% $HgCl_2$ (w/v) for 8 min followed by three washes with sterilized distiller water. For shoot and root development the stem segments were cultivated on basal MS medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose and 0.7% (w/v) agar. The *in vitro* growth occurred under aseptic controlled environmental conditions (16/8 h light/dark, 60 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetic photon flux density (PPFD), 25 °C, 60–70% relative air humidity). Fully expanded leaves from the 2nd or 3rd nodes of the stem were collected after four weeks of cultivation (Supplementary Fig. S1b).

Ex vitro acclimation

For further *ex vitro* acclimation, the *in vitro*-grown plantlets having 5–6 cm long regenerated shoots with 2–3 internodes and with a well-developed root system were removed from the culture tubes. The plants were transferred into plastic pots containing a mixture from sterile soil and peat. A one-month acclimation was maintained in a growth chamber (Nüve, TK 120, 16/8 h light/dark, 100 $\mu mol\ m^{-2}\ s^{-1}$ PPFD, 22 ± 2 °C, the relative humidity was decreased from 90% to 60% every week) (Supplementary Fig. S1c). Next, the acclimated plants were transferred to greenhouse for another month followed by transfer to normal garden soil in the field of Lozen mountain, near Sofia. After one year of acclimation to the field conditions, newly formed fully expanded leaves from the 2nd or 3rd nodes of the stem of *ex vitro* plants were harvested during blooming in May (Supplementary Fig. S1d). Note that the *in situ* and *ex vitro* plants were grown simultaneously (Supplementary Fig. S1a and d).

Flow cytometry

DNA ploidy level was checked by flow cytometry analysis according to Radziejwoski et al. (2011).

DNA isolation

DNA was isolated from *L. album* leaves according to Dellaporta et al. (1983).

Random amplified polymorphic DNA (RAPD) analysis

Six 10-mer oligonucleotides were used as primers for the RAPD analysis, and five of them generated reproducible results (Supplementary Table S1). A specialized RAPD kit (Ready To Go RAPD Analysis Beads, GE Healthcare UK Limited) was used for setting up the reaction according to the manufacturer's instructions in the following concentrations: 20 ng genomic DNA and 25 pmol primer in total amount of 25 μL. The reaction was performed in programmable thermal controller PTC-150 MiniCycler™, as follows: 96 °C for 2 min followed by 45 cycles of 96 °C for 1 min, 36 °C for