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The importance of applied light quality on the process of shoot organogenesis and production of phenolics and carbohydrates in *Lachenalia* sp. cultures *in vitro*

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ABSTRACT

Light may be particularly important for *in vitro* plant morphogenesis due to its effects on primary and secondary plant metabolism (sugars and phenolics). This is the first report on the formation of *Lachenalia* adventitious shoots (caulogenesis) in relation to the content of endogenous phenolic compounds and carbohydrates in *in vitro* cultures exposed to diverse light conditions. The cultures were established from microshoot explants cultivated on Murashige and Skoog medium (3% sucrose, benzylaminopurine 2.5 μM, naphthalene acetic acid 0.5 μM). Total soluble phenolics, free and conjugated phenolic acids (cinnamic, p-coumaric, caffeic, ferulic, sinapic, chlorogenic), soluble carbohydrates and starch content were estimated in adventitious shoots of lachenalia 'Rupert' and 'Ronina' developed under white, blue, red light and in darkness. White and blue light stimulated the formation of adventitious shoots and buds which had also higher total phenolics content than those formed under red light and in darkness. Red light limited the process of organogenesis of lachenalia 'Ronina'. Caffeic acid was the most abundant phenolic acid, particularly in the shoots grown under blue and white light. Low concentration of endogenous ferulic acid accompanied the formation of adventitious roots. Red light and darkness caused elongation of lachenalia shoots, which also contained more glucose or fructose than those grown under blue and white light.

1. Introduction

Lachenalia (*Lachenalia* Jacq.) is an ornamental bulb geophyte genus belonging to Asparagaceae family and including 133 species distributed mainly in the winter rainfall zone of Namibia and South Africa (Duncan, 2012). Hybridisation and selection research initiated fifty years ago resulted in obtaining improved cultivars with unusually diverse phenotypes featuring a broad range of shapes and color combinations (Kleynhans, 2006). *In vivo*, majority of lachenalia cultivars are characterized by poor propagation rate *via* adventitious bulbs. Due to lachenalia susceptibility to *Ornithogalum Mosaic Virus*, *in vitro* propagation of the breeding material is adapted using leaf explants that develop adventitious shoots (Niederwieser and Ndou, 2002). As the adventitious organogenesis is crucial to effective *in vitro* propagation, new ways of increasing the efficiency of adventitious shoots formation are constantly sought after. Adventitious shoots organogenesis (caulogenesis) in bulbous plants depends on many factors, both endogenous (*e.g.* type and topophysical position of the initial explant, genotype), and exogenous (*e.g.* medium composition or physical conditions of *in vitro* culture) (Bach and Sochacki, 2013). Light, one of the exogenous factors, may be particularly important for *in vitro* plant morphogenesis due to its effects on primary and secondary plant metabolism - sugars and phenolic compounds, respectively (Matthew et al., 2004; Hoshino and Cuello, 2006). Sugars as the products of primary metabolism are not only the source of energy but also play a role as signaling molecules, which can modulate photosynthesis, growth and plant development (Eckstein et al., 2012).

Studies on the effect of different light conditions on carbohydrate content in the shoots of bulbous plants are limited and according to our knowledge they have not been carried out in lachenalia *in vitro* culture yet. However, Du Toit et al. (2004) investigated carbohydrate content in various organs of lachenalia 'Ronina' during *in vivo* bulb formation, and Bach et al. (2015) described the relation between phenolic compounds and carbohydrates during lachenalia bulb formation *in vitro*.

Light is one of the most influential factors that may change plant secondary metabolism through a stimulatory effect on biosynthesis of phenolics by influencing activity of phenylalanine ammonia-lyase (PAL, the first enzyme in the phenylpropanoid pathway in which phenolics are produced) (Engelsma, 1974; Lefsrud, 2008). Phenolics are a diverse group of chemical compounds derived from phenylalanine and tyrosine that include flavonoids and phenolic acids (Dixon and Paiva, 1995). They have been reported to play both negative and positive role in

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organogenesis or callus development in plant tissue cultures. Numerous authors suggested inverse correlation between accumulation of phenolics and plant regeneration abilities during caulogenesis process, for example in *Sterculia urens* (Devi et al., 2013), *Tacitus bellus* (Mitrović et al., 2015), and *Cichorium intybus* (Legrand and Bouazza, 1991). On the other hand, phenolics stimulated callus growth in *Fragaria* × *ananassa* (Arnaldos et al., 2001), *Saccharum officinarum* (Lorenzo et al., 2001), or *Vitis vinifera* (Feucht et al., 1998).

The aim of this study was to evaluate adventitious organogenesis in lachenalia to find the optimal light conditions in the cultures. Additionally, on the basis of the above-mentioned contrary studies, we decided to examine the caulogenesis in terms of quality and quantity content of endogenous phenolics and carbohydrates in the shoot tissues.

2. Materials and methods

2.1. Plant material

In vitro cultures were established from donor lachenalia 'Rupert' and 'Ronina' plants obtained from Afriflowers company (South Africa). The initial explants - microshoots (basal parts 10–15 mm long of one-leaf shoots) were collected directly from the plants regenerated in a one year old *in vitro* culture exposed to white light, subcultured every eight weeks. Microshoots were placed vertically in 100 ml Erlenmeyer flasks on 25 ml of Murashige and Skoog medium (Murashige and Skoog, 1962) modified for adventitious shoots formation by supplementing with sucrose (3%), 2.5 μM benzylaminopurine and 0.5 μM naphthalene acetic acid. The medium pH was adjusted to 5.8 before adding 0.7% W/V Difco Bacto agar. Each flask contained five lachenalia initial explants and five flasks were exposed to each light condition. The cultures were transferred onto fresh medium every eight weeks. After two cycles of cultures (16 weeks), the plant material regenerated under different light conditions was evaluated. Degree of regeneration was assessed on the process of caulogenesis (number of buds and shoots) and on the number of occasionally appeared roots per a single initial explant. The length of shoots was also measured.

2.2. Light conditions

The initial explants were grown at 23/21 °C (day/night), in darkness or they were exposed to three different light spectrum (16 h photoperiod) provided by fluorescent lamps: white (390–760 nm, Tungsram lamps 40 W F33), red (647–770 nm, peak wave length 660 nm, Philips TLD 36 W), and blue (450–492 nm, peak wave length 450 nm, Philips TLD 36 W). The spectrum of white, blue and red light was measured with a portable LI-1800 spectroradiometer (LI-COR, USA). The average photosynthetic photon flux density at plant level was 30 $\mu mol\ m^{-2}\ s^{-1}$.

2.3. Samples preparation

Shoots samples were collected, immediately frozen in liquid nitrogen and lyophilised (LGA05, MLW, Leipzig, Germany, upgraded by JWE, Warsaw, Poland). Then samples were pulverized (MM400, Retsch, Haan, Germany) and this material was used for all analyses.

2.4. Phenolics extraction

About 20 mg exactly weighted samples were extracted to 0.5 ml of 2% acetic acid in methanol for 1 h at 250 rpm on a rotary shaker (JWE, Warsaw, Poland). Then the samples were centrifuged (15 min, 22,000 ×g, 10 °C) and the supernatant was collected. This extract was used later for total soluble phenolics content, free and conjugated phenolic acids estimation.

2.5. Total soluble phenolics content estimation

The total soluble phenolics content (TPC) was measured spectrophotometrically by means of Singleton method (Singleton et al., 1999). The extract aliquot (50 μl) was diluted in 0.5 ml of deionized water and 0.2 ml of Folin-Ciocalteu reagent (POCH, Gliwice, Poland) was added. After 10 min incubation, 0.7 ml saturated Na_2CO_3 was added to fix pH = 10. Samples were vortexed and after 2 h incubation, transferred to 96-well plates. Absorbance at 765 nm was read on a microplate reader (Synergy II, Biotek). Chlorogenic acid was used for calibration. The analysis was performed in five replicates. The results were expressed as milligram of chlorogenic acid equivalents per gram of dry weight (DW).

2.6. Free and conjugated phenolic acids estimation

The remained extract was diluted with deionized water to total volume of 2.5 ml. Then 100 μl concentrated H_3PO_4 was added and samples were shaken with 1 ml of ethyl acetate, the upper layer was collected then the liquid-liquid extraction was repeated. Collected organic layers were pooled and evaporated under an N_2 stream (Turbowap, Biotage, USA). Dry residue was resuspended in 0.4 ml of methanol and used for free phenolic acids estimation. The remaining aqueous layer containing bounded phenolic acids was hydrolysed after addition of 250 μl of concentrated HCl (at 85 °C for 30 min). The released phenolic acids were extracted the same way as their free fraction. After evaporation dry residue was redissolved in 0.4 ml of methanol and used for conjugated phenolic acids estimation. All samples were filtered (0.22 μm nylon membrane) and analyzed by HPLC. Analyses were done on an Agilent 1200 system with fluorescence detector (FLD). Separation of phenolics was achieved on Zorbax Eclipse XDB-C18 4.6 × 75 mm 3.5 μm analytical column, and under linear gradient of A: methanol and B: water with 2% acetic acid, at 0 min 5% A, to 35 min 70% A. The optimal parameters of fluorescence detection were chosen according to the absorption and emission spectra taken for standards online. The analyses were carried out in five replicates. The results were expressed in microgram per gram of dry weight (DW).

2.7. Soluble sugars estimation

Soluble sugars were estimated by HPLC as detailed described by Pociecha et al. (2016). Samples were extracted in DI water and after dilution separated by HPLC (Agilent 1200) in anion exchange mod with pulse amperometricaly detection. Glucose, fructose and sucrose were measured and expressed in milligram per gram of DW. The analysis of soluble sugars was performed in triplicate.

2.8. Starch estimation

Starch was determined in pellets remaining after soluble sugars analysis with the use of enzymatic hydrolysis (sequentially applied alpha-amylase and amyloglucosidase). Alpha-amylase (Sigma) in 50 mM potassium phosphate buffer pH 6.9, with 6.7 mM of NaCl (PBS), and amyloglucosidase (Sigma) in 200 mM sodium acetate buffer pH 4.5 were used. The pellets were rinsed with ultrapure water then 350 μl of alpha-amylase solution (0.2 mg in 25 ml PBS) was added, the samples were vortexed and placed in a boiling water bath for 10 min. The samples were allowed to cool before 450 μl of amyloglucosidase was added (1 mg in 30 ml of acetate buffer), and the samples were placed in a 50 °C water bath for 1 h. Released glucose was measured spectrophotometrically by phenol sulfuric acid method (Bach et al., 2015) and expressed in milligram per gram of DW. The analyses of the starch were performed in triplicate.