Growth Regulation and Validation of Homogeneity in In Vitro-Derived Bleeding Heart by Molecular Markers and Spectral Analysis of Pigments

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Abstract

The aim of this study is to analyze the morphogenetic events in *Lamprocapnos spectabilis* (L.) Fukuhara (bleeding heart) ‘Gold Heart’ and ‘White Gold’ micropropagated in various culture media, and to evaluate the biochemical and genetic stability of the produced plants. None of the sixteen tested plant growth regulator (PGR) combinations improved the micropropagation efficiency or morphological traits of shoots in ‘Gold Heart’ compared to the PGRs-free control, indicating low intrinsic regenerability of this cultivar. As for bleeding heart ‘White Gold’, culture medium with 0.5 mg·L⁻¹ IBA and 2.0 mg·L⁻¹ BA was optimal in terms of multiplication ratio (9.8) and shoot parameters. Addition of PGRs was necessary for effective rooting in both cultivars studied. Some experimental combinations declined the concentration of chlorophylls and carotenoids in the shoots of bleeding heart, especially if a significant amount of callus was formed at the shoot base. The PGR-supplemented media stimulated the abundant secretion of phenols by shoots. The survival rate and quality of the plantlets after acclimatization varied from 0.0 to 100%, depending on the composition of the multiplication medium. Plants with a higher number of leaves were more likely to survive acclimatization. Somaclonal variation was detected in 36.8 to even 69.1% of in vitro-derived plants by two genetic marker systems. Most of that variation was irrelevant, however, some genotypes were significantly different from the predominant control.

Keywords Acclimatization · Metabolism · Micropropagation · Plant growth regulators · Plant quality · Somaclonal variation

Introduction

Lamprocapnos spectabilis (L.) Fukuhara is a plant species of the Fumariaceae botanical family, well-known for its exceptional heart-shaped red, white, or pink flowers. The species is among the most valuable in the floristic market. It has also potential in landscape architecture; in gardens and parks worldwide (Hodges 2012). Moreover, the species is valued in medicine and cosmetology (Iwasa and Kim 1997; McNulty et al. 2007). The development of horticulture allowed to easily force the blooming of bleeding heart any time of the year (Hodges 2012). However, the efficiency of

traditional reproduction via root cuttings is limited by the low number of cuttings produced or unfavorable biotic and abiotic conditions during cultivation. They can negatively affect not only the yield size but also the content of active components found in bleeding heart (mainly isoquinolines) and, thus, the quality of the plant material. Therefore, for commercial production, it is necessary to develop a more effective, tissue-culture-based protocol.

Approximately 200 plant genera (mostly ornamentals) are now produced on a mass scale in commercial laboratories worldwide (Kulus 2015a). Despite they are critical to the species commercial success, there are just a few studies related to tissue culture systems in bleeding heart, related mostly to somatic embryogenesis (Lee and Lee 2003; Lee et al. 2004). Somatic embryogenesis (SE), however, is a complex process facing several bottlenecks reported also with bleeding heart, e.g., asynchronous development of embryos, difficulties with embryo maturation, and conversion into complete plantlets (Kulus 2020a). Moreover, SE

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may not be a genetic stability guarantee if a callus phase is involved, while a suspension culture system is susceptible to contamination development and requires expensive bioreactors (Joshi and Kumar 2013). Consequently, developing a rapid and easy-to-perform clonal micropropagation protocol is also important.

Meristematic explants; such as shoot tips, shoot apices, or nodal segments; produce less variation in vitro than highly differentiated tissues such as roots, leaves, and stems (Krishna et al. 2016). Activation of axillary buds located in the leaf axil can provide a high multiplication ratio in a short time (Grzegorzczak-Karolak et al. 2015). In contrast to SE performed in liquid culture systems, propagation via axillary bud activation is quite easy to perform and the shoots are less prone to hyperhydration occurrence. This is especially important during acclimatization to ex vitro conditions.

Composition of the culture medium is a vital element affecting the efficiency of a micropropagation protocol. Auxins and cytokinins are usually indispensable for the induction of de novo regeneration in plants cultured in vitro (Gaspar et al. 1996). Among several other functions, auxins are usually involved in the induction of embryogenesis, rhizogenesis, and callogenesis, while cytokinins stimulate the proliferation of shoots (Simon and Petrášek 2011; Dobránszki and Mendler-Drienyovszki 2014). Plant hormones, however, rarely work alone and a simultaneous or sequential combination of various PGRs is necessary to achieve a high-scale propagation ratio. The optimal composition of the culture medium depends on the genotype, explant parameters, and desired type of morphogenetic response.

PGRs affect not only the regeneration of plant organs, and their further acclimatization ability, but also the biosynthetic pathway of primary and secondary metabolites, which might be of significant importance to the pharmacological industry (Lucchesini et al. 2009). For example, Grzegorzczak-Karolak et al. (2015, 2016) found that the in vitro culture of *Scutellaria alpina* L. and *Scutellaria altissima* L. can be an effective system for the overproduction of bioactive compounds with different pharmacological activity during long-term subculturing. In contrast, studies on exploiting the potential of in vitro cultures in producing valuable metabolites in plants of the Fumariaceae family are still underdeveloped.

Another important issue related to plant tissue culture is the so-called somaclonal variation, i.e., genetic and/or phenotype variation induced by in vitro culture conditions. This phenomenon usually occurs in response to a high concentration of PGRs, which induce the formation of callus; a highly unstable *quasi*-tissue. Somaclonal variation is generally unwanted in the reproduction of elite plant material. It can be useful, though, in breeding programs as reported with chrysanthemum (Miler and Zalewska 2014). Currently, there is no information on the stability of *L. spectabilis* reproduced in vitro. Therefore,

analyses of its genetic integrity are important when developing a mass-scale micropropagation protocol.

The aim of this research was to analyze the morphogenetic events in two popular cultivars of *Lamprocapnos spectabilis* ('Gold Heart' and 'White Gold') micropropagated in culture media with various combinations of PGRs. The stability of the produced plantlets was verified with biochemical and molecular markers. The viability and quality of the ex vitro-transferred plants were also evaluated.

Materials and Methods

Basal Medium and Physical Conditions in the Growth Chamber

The Murashige and Skoog (1962) medium (MS) modified by increasing by half the concentration of calcium II chloride ($\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$), iron sulfate (FeSO_4) and $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, supplemented with 0.09 M sucrose, and solidified with 0.8% (w/v) agar (Biocorp, Poland) was used in the experiment. The pH was adjusted to 5.8 (with 0.1 M HCl and 0.1 M NaOH; Chempur, Poland) after adding all media components (Chemia, Poland), before sterilization at 105 kPa and 121 °C for 20 min. The medium (40 mL) was poured into 350-mL vessels and sealed with plastic caps.

The cultures were grown in the growth chamber at 24 ± 1 °C, under 16-h photoperiod conditions and photosynthetic photon flux density (PPFD) of $29.8 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ provided by standard cool daylight TLD 54/36 W fluorescent tubes with a color temperature of 6200 K (Koninklijke Philips Electronics N.V., the Netherlands).

Biological Material

In vitro-derived shoots of *Lamprocapnos spectabilis* (L.) Fukuhara 'Gold Heart' and 'White Gold' were used as the donor plants. Axenic cultures of the two cultivars were obtained from the in vitro gene bank of the Laboratory of Ornamental Plants and Vegetable Crops, Faculty of Agriculture and Biotechnology, UTP University of Science and Technology, Bydgoszcz, Poland.

To produce the required number of explants, the donor plants were cloned via the single-node method in the modified PGRs-free MS medium (as above) in two propagation cycles, i.e., for about five months.

Effect of Various Combinations of Growth Regulators on the Multiplication and Morphogenetic Response of Explants

The 10-week-old shoots were cut into nodal segments, 5–10 mm-in-length (Fig. 1a), without leaves and inoculated

vertically in the modified MS medium; four explants per jar. Indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) were combined with 6-benzyladenine (BA) or 6-furfurylaminopurine (kinetin, KIN), each at 0.5 or 2.0 mg·L⁻¹. All PGRs were provided by Sigma-Aldrich®, USA, a PGRs-free control was also considered. A total of 17 experimental combinations were included (Table 1). The in vitro cultures were cultivated for eight weeks. One jar was considered a single repetition, the experiment was repeated thrice.

Morphological Analysis of Plants and Micropropagation Efficiency Evaluation

After a single propagation cycle, several morphological parameters were evaluated, including the mean number of shoots and leaves regenerated from a single inoculated explant, as well as the length [mm], fresh (FW) [mg], and

dry weight (DW) [%] of shoots. The multiplication ratio was assessed by counting the number of secondary explants (nodal segments) that could be easily excised from the recovered shoots. Also, the rooting efficiency [%], number, and length [mm] of roots were included. Moreover, callus regeneration frequency [%], its fresh [mg], and dry weight [%] were estimated. To analyze the dry weight, shoots or calli were desiccated in a laboratory oven at 105 °C for 180 min.

Biochemical Array

Spectrophotometric analysis of in vitro-grown plant pigments, extracted from at least five 100-mg-fresh-shoot samples, from every experimental combination was performed. The extraction of chlorophylls and carotenoids was performed with the method described by Lichtenthaler (1987) with 100% acetone. The spectrophotometric analysis of

Fig. 1 Micropropagation and acclimatization of *Lamprocapnos spectabilis*. **a** single-node explant used in the experiment; **b** callogenesis and proliferation of axillary shoots in bleeding heart GH cultured in MS medium with 2.0 mg·L⁻¹ IAA and 0.5 mg·L⁻¹ BA after 5 weeks of culture; **c** fully developed shoots of bleeding heart GH produced in MS medium with 0.5 mg·L⁻¹ IAA and 0.5 mg·L⁻¹ BA after 8 weeks

of culture; **d** rooted microshoot of bleeding heart GH prepared for acclimatization after 10 weeks of culture; **e** bleeding heart plants with different origin and quality after two months of ex vitro growth. BA 6-benzyladenine, GH ‘Gold Heart’, IAA indole-3-acetic acid, IBA indole-3-butyric acid, KIN kinetin, MS Murashige and Skoog (1962), WG ‘White Gold’. bar = 1 mm (a, b) or 1 cm (c–e)

Table 1 Influence of culture medium composition on the mean number of shoots and leaves formed from a single-node explant, shoot length [mm], multiplication ratio, and callus regeneration frequency [%]

PGR* concentration [mg·L ⁻¹]				Shoot mean number	Leaf mean number	Shoot length [mm]	Multiplication ratio	Callus [%]
IAA	IBA	BA	KIN					
Gold Heart								
–	–	–	–	1.9±0.4 a ¹	12.6±1.9 ab	24.0±2.6 a-d	5.1±0.9 ab	0.0 d
0.5	–	0.5	–	1.9±0.5 a	15.8±2.4 a	26.3±1.8 a-c	5.9±1.0 a	100 a
0.5	–	2.0	–	1.9±0.4 a	10.3±1.5 ab	14.8±2.3 c-e	4.2±0.8 ab	100 a
0.5	–	–	0.5	1.5±0.2 a	8.0±1.0 b	14.0±1.6 de	3.1±0.4 ab	50.0 bc
0.5	–	–	2.0	2.0±0.2 a	11.8±1.0 ab	15.1±1.1 c-e	4.4±0.5 ab	87.5 ab
2.0	–	0.5	–	1.3±0.2 a	10.5±1.9 ab	28.1±4.2 a	4.0±0.7 ab	100 a
2.0	–	2.0	–	2.0±0.3 a	11.3±1.8 ab	9.6±1.0 e	4.3±0.7 ab	100 a
2.0	–	–	0.5	1.3±0.2 a	6.3±0.4 b	22.3±1.2 a-d	2.5±0.3 b	0.0 d
2.0	–	–	2.0	1.9±0.4 a	12.8±1.4 ab	16.3±2.0 b-e	5.0±0.7 ab	0.0 d
–	0.5	0.5	–	1.6±0.3 a	10.7±2.4 ab	22.7±2.2 a-d	4.1±1.0 ab	100 a
–	0.5	2.0	–	2.1±0.3 a	11.8±1.5 ab	14.9±1.2 c-e	4.9±0.9 ab	100 a
–	0.5	–	0.5	1.3±0.2 a	6.9±0.7 b	19.9±1.5 a-e	2.8±0.5 ab	0.0 d
–	0.5	–	2.0	1.1±0.1 a	7.5±1.0 b	25.1±1.4 a-d	3.2±0.4 ab	12.5 cd
–	2.0	0.5	–	1.8±0.3 a	11.3±ab	26.9±3.1 ab	4.3±0.9 ab	100 a
–	2.0	2.0	–	1.8±0.4 a	6.8±1.2 b	8.2±2.1 e	2.6±0.3 ab	100 a
–	2.0	–	0.5	1.1±0.1 a	6.1±0.7 b	26.0±3.0 a-c	2.8±0.4 ab	100 a
–	2.0	–	2.0	2.0±0.4 a	10.5±2.0 ab	9.8±3.8 e	4.8±1.2 ab	50.0 bc
White Gold								
–	–	–	–	1.1±0.1 b	8.3±1.5 b	17.0±1.5 a-c	2.8±0.5 b	0.0 c
0.5	–	0.5	–	2.6±0.4 ab	14.6±1.9 ab	17.0±1.6 a-c	6.3±0.8 ab	0.0 c
0.5	–	2.0	–	2.6±0.3 ab	8.8±0.7 b	6.4±0.7 f	3.6±0.3 b	100 a
0.5	–	–	0.5	1.4±0.3 b	8.4±0.5 b	16.0±1.3 a-d	3.3±0.3 b	0.0 c
0.5	–	–	2.0	2.0±0.4 b	10.9±1.4 b	13.6±1.1 c-e	5.0±0.7 b	0.0 c
2.0	–	0.5	–	1.9±0.3 b	11.6±1.4 b	15.1±1.2 a-e	5.0±0.7 b	0.0 c
2.0	–	2.0	–	2.0±0.3 b	14.5±1.3 ab	10.0±0.5 d-f	5.3±0.4 b	100 a
2.0	–	–	0.5	1.0±0.0 b	7.1±0.4 b	17.0±0.8 a-c	2.6±0.2 b	0.0 c
2.0	–	–	2.0	1.9±0.2 b	11.5±1.9 b	14.9±0.5 a-e	4.6±0.7 b	0.0 c
–	0.5	0.5	–	1.8±0.2 b	12.1±1.0 b	14.6±1.3 a-e	5.3±0.5 b	100 a
–	0.5	2.0	–	4.0±1.0 a	22.1±5.1 a	14.4±3.4 b-e	9.8±2.4 a	100 a
–	0.5	–	0.5	1.4±0.2 b	8.5±0.6 b	16.8±1.0 a-d	3.0±0.3 b	0.0 c
–	0.5	–	2.0	1.3±0.2 b	9.5±0.8 b	19.3±1.9 a-c	3.7±0.3 b	50.0 b
–	2.0	0.5	–	1.7±0.3 b	10.4±1.7 b	15.9±1.1 a-d	4.1±0.8 b	100 a
–	2.0	2.0	–	1.3±0.2 b	7.1±1.0 b	8.3±1.1 ef	2.4±0.3 b	100 a
–	2.0	–	0.5	1.7±0.3 b	10.7±2.2 b	21.3±1.3 ab	4.5±0.8 b	87.5 a
–	2.0	–	2.0	1.6±0.3 b	10.1±1.3 b	21.4±1.3 a	4.1±0.8 b	100 a

BA 6-benzyladenine, IAA indole-3-acetic acid, IBA indole-3-butyric acid, KIN kinetin, PGR plant growth regulator

¹ Means ± standard errors in columns marked with the same letter do not differ significantly according to the HSD Tukey's test ($P \leq 0.05$);

extracts was performed in the spectrophotometer (Smart-Spec Plus™ BioRad, US).

Absorption maxima were defined for pigment-specific wavelengths ($\lambda_{\max}$): for carotenoids at 470 nm, and for chlorophyll *a* and *b* at 645 and 662 nm, respectively. The content of chlorophylls and carotenoids per gram of fresh matter of

shoots was calculated with the algebraic method following Lichtenthaler and Buschmann (2001).

Genetic Stability Evaluation

The genetic fidelity of in vitro-propagated *L. spectabilis* 'White Gold' shoots was assessed using randomly

amplified polymorphic DNA (RAPD) and start codon target polymorphism (SCoT) markers. A total of 68 specimens (four from each experimental combination) were included in the analysis. Total genomic DNA was isolated from fresh tissues using a Genomic Mini AX Plant Spin kit (A&A Biotechnology, Poland), according to the manufacturer's instruction. The isolated DNA was stored at 4 °C.

A total of 20 primers (10 RAPD and 10 SCoT, Genomed, Poland, Table 7) were used for the PCR reaction. PCR was performed in a BioRad C1000 Touch thermal cycler with heated cover (Bio-Rad, USA) in the 25- μ L reaction solution. The composition of the reaction solution, PCR profiles, and electrophoretic separation of amplified DNA fragments were described earlier in Kulus (2020b; Open Access). Gel images were recorded using a GelDoc XR + Gel Photodocumentation System (Bio-Rad, USA) UV transilluminator with Image Lab 4.1 software. Molecular weights of the fragments were estimated using a 100 – 3,000 bp DNA molecular marker (100 bp DNA Ladder H3 RTU, Nippon Genetics Europe GmbH, Germany).

The banding patterns were recorded as 0–1 binary matrices, where “1” indicates the presence and “0” the absence of a given fragment followed by statistical analysis. For every primer tested the total number of bands, monomorphic, polymorphic (present in the electrophoretic profile of more than one individual) and specific/unique *loci* (present in the electrophoretic profile of a single individual) was counted.

In Vitro Rooting and Acclimatization to Ex Vitro Conditions

Five shoots from each experimental combination were rooted and acclimatized. For this purpose, the ‘Gold Heart’ and ‘White Gold’ shoots were dissected and transferred on the rooting MS medium containing half-strength macronutrients ($\frac{1}{2}$ MS), 2% sucrose, and 1.0 mg·L⁻¹ 1-naphthaleneacetic acid (NAA) for 14 days.

Acclimatization was conducted from July 15 to July 29 in natural light conditions in a glasshouse at the temperature 19.5 – 27.3 °C (mean 23.1 °C) and 59 – 76% relative humidity (mean 66.7%). Bleeding hearts were grown in plastic trays filled with a mixture of peat and perlite (2:1), sprayed with water, and covered with perforated foil and geo-cover. The acclimatization effectiveness, i.e., survival rate [%] and quality of plants, was assessed after 14 days. Plants that remained in the unchanged healthy form were considered fair quality. Those, which survived acclimatization but were tiny or had withered leaves after two weeks were considered poor quality. The quality of shoots

with evident signs of growth, i.e., development of new healthy leaves, was considered superb. After one month, the plants were transferred to individual plastic pots filled with the same substrate and planted in a permanent place on benches.

Statistical Analysis

The single-factor experiment was performed in a completely randomized design. The Freeman-Tukey transformation was used for the data expressed as a percentage. The results were statistically analyzed with one-way analysis of variance (ANOVA), and the comparisons of means were made with HSD Tukey's Multiple Comparison Test ($P \leq 0.05$) using Statistica 12.0 and ANALWAR-5.2-FR tools. Tables with results provide real numerical data, while alphabet letters point to homogenous groups, following the statistical calculations based on transformed data.

Associations between the analyzed traits of plants were analyzed, based on the Pearson correlation coefficient (r ; $P \leq 0.05$). The associations were considered as moderate ($0.3 < |r| \leq 0.5$), strong ($0.5 < |r| \leq 0.7$) or very strong ($0.7 < |r| \leq 1.0$). Low correlations ($|r| \leq 0.3$) were not included.

As for the molecular analysis, Agglomerative Hierarchical Clustering with Unweighted Pair-Group Average Method (AHC UPGMA) was used to draw the dendrograms. GeneAEx 6.5 software (Peakall and Smouse 2012) was used to perform the principal component analysis (PCoA). Population groups were distinguished based on the analysis of molecular variance (AMOVA) estimates.

Results

Effect of PGRs Combinations on the Multiplication and Morphogenetic Response of Explants

The effect of plant growth regulators on the morphogenetic response of explants was cultivar-specific (Table 1). Caulogenesis was evident in the third week of the in vitro culture. There was no influence of culture medium composition on the number of shoots produced by a ‘Gold Heart’ explant (1.1 – 2.1). The number of leaves developed by a single explant in this cultivar varied from 6.1 (MS with 2.0 mg·L⁻¹ IBA and 0.5 mg·L⁻¹ KIN) to 15.8 (MS with 0.5 mg·L⁻¹ IAA and 0.5 mg·L⁻¹ BA). The longest shoots were produced on the MS medium with 2.0 mg·L⁻¹ IAA and 0.5 mg·L⁻¹ BA (28.1 mm). None of the PGRs combinations, however, improved the parameters of shoots compared to the PGRs-free control. The highest number of offspring explants produced from a single inoculated nodal segment (5.9) was dissected after applying the medium with 0.5 mg·L⁻¹ IAA and 0.5 mg·L⁻¹ BA. Most of the ‘Gold Heart’ explants

(50.0 – 100%) formed callus at the base of shoots (Fig. 1b–c), except for those cultured on the control medium and in the presence of 2.0 mg·L⁻¹ IAA and 0.5 or 2.0 mg·L⁻¹ KIN or 0.5 mg·L⁻¹ IBA and 0.5 or 2.0 mg·L⁻¹ KIN (Table 1).

As for the ‘White Gold’ cultivar, the highest number of shoots regenerated by a single inoculated explant (4.0) was found on the MS medium with 0.5 mg·L⁻¹ IBA and 2.0 mg·L⁻¹ BA, and it was nearly fourfold higher than in the control medium devoid of auxins and cytokinins (Table 1). This PGRs combination also increased the number of leaves produced (22.1) compared to most other treatments (7.1 – 12.1), and the multiplication ratio (9.8). The longest shoots (over 21 mm) were found in the medium with 2.0 mg·L⁻¹ IBA and 0.5 or 2.0 mg·L⁻¹ KIN, although they were not statistically different from the control shoots (17.0 mm). On the other hand, media with 0.5 mg·L⁻¹ IAA and 2.0 mg·L⁻¹ BA, as well as 2.0 mg·L⁻¹ IBA and 2.0 mg·L⁻¹ BA suppressed the elongation of shoots (Table 1). No callogenesis was reported in the control medium, but also in several other combinations containing auxins (mainly IAA) and cytokinins (Table 1).

In *L. spectabilis* ‘Gold Heart’, the highest fresh weight of shoots was reported in the medium with 2.0 mg·L⁻¹ IBA and 2.0 mg·L⁻¹ KIN (249.1 mg), although it was not statistically different from the shoots grown on the PGRs-free control (144.6 mg). Application of media fortified with 2.0 mg·L⁻¹ IAA and 2.0 mg·L⁻¹ BA, 2.0 mg·L⁻¹ IBA and 2.0 mg·L⁻¹ BA, as well as 2.0 mg·L⁻¹ IBA and 2.0 mg·L⁻¹ KIN led to a significant decrease in the share of dry weight in shoots (4.6 – 7.1%), compared to the other treatments (15.5 – 21.5%). On the other hand, the highest fresh weight of callus was found in the experimental combinations with 2.0 mg·L⁻¹ IBA and 0.5 or 2.0 mg·L⁻¹ BA (336.2 – 394.9 mg). Only the medium with 2.0 mg·L⁻¹ IAA and 0.5 mg·L⁻¹ BA led to a significant decline in the share of DW in the fresh callus mass (7.8%) (Table 2).

As for bleeding heart ‘White Gold’, the highest FW in shoots were reported after using the medium fortified with 0.5 mg·L⁻¹ IAA and 0.5 mg·L⁻¹ BA or 0.5 mg·L⁻¹ IBA and 2.0 mg·L⁻¹ BA (336.2 – 352.0 mg). On the other hand, the highest and lowest share of DW in the fresh shoot weight was found in the media with 0.5 mg·L⁻¹ IBA and 2.0 mg·L⁻¹ KIN (29.0%) and 2.0 mg·L⁻¹ IBA and 0.5 mg·L⁻¹ BA (7.7%), respectively. MS medium fortified with 0.5 mg·L⁻¹ IBA and 2.0 mg·L⁻¹ BA stimulated the most intensive callus formation (427.6 mg FW per inoculated explant). Nonetheless, there was no influence of culture medium composition on the share of DW in the fresh callus mass 13.6 – 23.5%) (Table 2).

The highest rooting efficiency in the ‘Gold Heart’ cultivar was reported with 0.5 mg·L⁻¹ IAA and 0.5 mg·L⁻¹ KIN (62.5% rooting rate). Also, 37.5% shoots grown in the control medium regenerated roots (Table 3). The other

experimental combinations were less effective in stimulating rhizogenesis (0.0 – 14.3% rooting rate). The highest number of roots produced by a single rooted explant (3.0 – 4.0) was reported in the media supplemented with 0.5 or 2.0 mg·L⁻¹ IBA and 0.5 mg·L⁻¹ KIN. Explants cultured in those two media and in the PGRs-free control regenerated the longest roots (48.0 – 64.0 mm) (Table 3).

The MS medium fortified with 0.5 mg·L⁻¹ IBA and 0.5 mg·L⁻¹ KIN was superior in terms of root development in the ‘White Gold’ cultivar (Table 3). This combination stimulated rhizogenesis in 37.5% explants, with 2.3 roots per explant and the greatest elongation of roots (35.3 mm). Also, the medium with 2.0 mg·L⁻¹ IAA and 2.0 mg·L⁻¹ KIN provided good rooting parameters in this cultivar. Application of 2.0 mg·L⁻¹ IBA and 0.5 mg·L⁻¹ KIN resulted in the highest rooting rate (42.9%), but the produced roots were shorter (9.2 mm) than from the previously described media (Table 3).

Effect of Auxins and Cytokinins on the Biosynthesis of Pigments in Shoots of Bleeding Heart

Medium composition had a significant influence on the biosynthesis of pigments in *L. spectabilis* (Table 4). The concentration of chlorophyll *a* in the shoots of bleeding heart ‘Gold Heart’ was markedly reduced by the experimental combination containing 2.0 mg·L⁻¹ IBA and 2.0 mg·L⁻¹ KIN (0.08 mg·g⁻¹ fresh tissue). The highest concentration of chlorophyll *b* was found in the shoots grown in the medium with 0.5 mg·L⁻¹ IAA and 2.0 mg·L⁻¹ KIN (0.26 mg·g⁻¹), while the lowest in 0.5 mg·L⁻¹ IAA and 2.0 mg·L⁻¹ BA (0.08 mg·g⁻¹). The ratio of chlorophyll *a*: *b* usually had a value > 1.0. Only in the shoots of bleeding heart ‘Gold Heart’ grown in the presence of 2.0 mg·L⁻¹ IBA and 2.0 mg·L⁻¹ KIN, the concentration of chlorophyll *b* (0.12 mg·g⁻¹) was higher than of chlorophyll *a* (0.08 mg·g⁻¹). This medium composition also negatively affected the biosynthesis of carotenoids (0.014 mg·g⁻¹) compared to most other treatments (0.065 – 0.113 mg·g⁻¹). Some PGR combinations enhanced the production of the analyzed compounds in the ‘Gold Heart’ cultivar; by even 46.7% (chlorophyll *a*), 73.3% (chlorophyll *b*), or 47.1% (carotenoids); compared to the control. Those differences, however, were statistically non-significant according to the HSD Tukey’s test (Table 4).

Application of MS medium containing 0.5 or 2.0 mg·L⁻¹ IAA and 2.0 mg·L⁻¹ BA contributed to a decrease in the content of chlorophyll *a*, the ratio of chlorophyll *a*: *b*, and concentration of carotenoids in the shoots of bleeding heart ‘White Gold’ (Table 4). On the other hand, the highest and lowest concentration of chlorophyll *b* was found when using media with 0.5 mg·L⁻¹ IAA and 0.5 mg·L⁻¹ KIN (0.21 mg·g⁻¹ fresh tissue) and 0.5 mg·L⁻¹ IAA and 0.5 mg·L⁻¹ BA (0.11 mg·g⁻¹), respectively. Similarly to

Table 2 Influence of culture medium composition on the fresh (FW) and dry weight (DW) [mg and %, respectively] in shoots and calli obtained from a single inoculated explant of *Lamprocapnos spectabilis*

PGR* concentration [mg·L ⁻¹]				Shoot FW [mg]	Share of DW in shoot [%]	Callus FW [mg]	Share of DW in callus [%]
IAA	IBA	BA	KIN				
Gold Heart							
–	–	–	–	144.6±24.3 ab	20.1 a	0.0 g	–
0.5	–	0.5	–	96.5±20.3 b	9.1 ab	104.4±24.6 df	10.6 ab
0.5	–	2.0	–	44.9±6.5 b	16.0 a	219.7±53.2 b-d	17.9 ab
0.5	–	–	0.5	63.6±10.8 b	18.4 a	10.5±5.5 fg	21.0 ab
0.5	–	–	2.0	60.7±15.1 b	15.5 a	18.5±3.5 ef	17.3 ab
2.0	–	0.5	–	78.7±17.0 b	18.8 a	75.8±25.8 ef	7.8 b
2.0	–	2.0	–	33.7±7.1 b	7.1 b	241.0±41.9 b-d	12.4 ab
2.0	–	–	0.5	92.5±10.8 b	21.4 a	0.0 g	–
2.0	–	–	2.0	106.9±28.1 b	21.5 a	0.0 g	–
–	0.5	0.5	–	71.0±15.3 b	19.4 a	150.5±22.9 c-e	16.9 ab
–	0.5	2.0	–	57.5±19.5 b	15.5 a	271.1±46.3 a-c	16.7 ab
–	0.5	–	0.5	98.9±10.7 b	19.3 a	0.0 g	–
–	0.5	–	2.0	118.7±9.2 b	17.5 a	8.6±4.3 fg	11.6 ab
–	2.0	0.5	–	116.1±22.2 b	18.9 a	394.9±39.0 a	20.9 ab
–	2.0	2.0	–	28.5±6.0 b	4.6 b	336.2±42.5 ab	15.9 ab
–	2.0	–	0.5	73.3±15.8 b	16.2 a	114.7±11.0 d-f	12.0 ab
–	2.0	–	2.0	249.1±69.7 a	5.5 b	63.0±31.4 ef	39.4 a
White Gold							
–	–	–	–	49.8±6.3 ab	23.3 ab	0.0 e	–
0.5	–	0.5	–	352.0±101.1 a	12.2 ab	0.0 e	–
0.5	–	2.0	–	55.7±10.5 ab	9.0 ab	280.0±44.9 ab	16.5 a
0.5	–	–	0.5	71.5±6.7 ab	18.2 ab	0.0 e	–
0.5	–	–	2.0	73.6±8.7 ab	24.7 ab	0.0 e	–
2.0	–	0.5	–	95.7±17.7 ab	18.9 ab	0.0 e	–
2.0	–	2.0	–	80.5±11.8 ab	13.3 ab	189.9±32.8 bc	13.6 a
2.0	–	–	0.5	66.8±5.3 ab	22.6 ab	0.0 e	–
2.0	–	–	2.0	94.6±14.3 ab	23.4 ab	0.0 e	–
–	0.5	0.5	–	49.2±4.4 ab	13.8 ab	117.5±20.3 b-d	19.7 a
–	0.5	2.0	–	336.2±163.4 a	20.1 ab	427.6±128.5 a	15.2 a
–	0.5	–	0.5	92.0±5.4 ab	17.6 ab	0.0 e	–
–	0.5	–	2.0	78.9±19.5 ab	29.0 a	67.7±22.2 cd	12.1 a
–	2.0	0.5	–	46.8±10.1 ab	7.7 b	115.6±14.7 b-d	15.0 a
–	2.0	2.0	–	26.8±5.2 b	9.3 ab	173.5±34.1 b-d	19.8 a
–	2.0	–	0.5	66.0±11.7 ab	19.7 ab	180.3±21.0 b-d	16.7 a
–	2.0	–	2.0	86.1±10.0 ab	25.7 ab	139.2±30.7 b-d	23.5 a

¹ Means ± standard errors in columns marked with the same letter do not differ significantly according to the HSD Tukey's test ($P \leq 0.05$)

*BA 6-benzyladenine, IAA indole-3-acetic acid, IBA indole-3-butyric acid, KIN kinetin, PGR plant growth regulator

‘Gold Heart’, also in the ‘White Gold’ cultivar, shoots grown in some PGRs-fortified media had a slightly higher content of metabolites than the control ones. For example, shoots cultured in the medium with 0.5 mg·L⁻¹ IAA and 0.5 mg·L⁻¹ KIN produced 46.2% more chlorophyll *a*, 50% more chlorophyll *b*, and 36.4% more carotenoids. This tendency, however, was not significant according to Tukey's test (Table 4).

It was also found that explants cultured on the media supplemented with auxins and cytokinins secreted abundantly phenols, resulting in the browning of media already in the first month of in vitro culture. This phenomenon was not observed with the control medium.

Table 3 Influence of culture medium composition on the rhizogenesis efficiency [%], number, and length [mm] of roots per rooted explant

PGR* concentration [mg·L ⁻¹]				Rooting [%]	No. of roots per rooted explant	Root length [mm]
IAA	IBA	BA	KIN			
Gold Heart						
–	–	–	–	37.5 ab ¹	2.0±0.6 bc	64.0±26.2 a
0.5	–	0.5	–	0.0 b	–	–
0.5	–	2.0	–	0.0 b	–	–
0.5	–	–	0.5	62.5 a	1.2±0.3 c	22.0±2.5 bc
0.5	–	–	2.0	0.0 b	–	–
2.0	–	0.5	–	0.0 b	–	–
2.0	–	2.0	–	0.0 b	–	–
2.0	–	–	0.5	0.0 b	–	–
2.0	–	–	2.0	0.0 b	–	–
–	0.5	0.5	–	0.0 b	–	–
–	0.5	2.0	–	12.5 b	1.0±0.0 c	8.0±0.0 c
–	0.5	–	0.5	12.5 b	4.0±0.0 a	48.0±4.0 a-c
–	0.5	–	2.0	0.0 b	–	–
–	2.0	0.5	–	14.3 b	2.0±0.0 bc	21.0±1.0 bc
–	2.0	2.0	–	0.0 b	–	–
–	2.0	–	0.5	12.5 b	3.0±0.0 ab	60.0±0.0 ab
–	2.0	–	2.0	0.0 b	–	–
White Gold						
–	–	–	–	0.0 c	–	–
0.5	–	0.5	–	0.0 c	–	–
0.5	–	2.0	–	0.0 c	–	–
0.5	–	–	0.5	12.5 bc	1.0±0.0 b	3.0±0.0 b
0.5	–	–	2.0	12.5 bc	1.0±0.0 b	5.0±0.0 b
2.0	–	0.5	–	0.0 c	–	–
2.0	–	2.0	–	0.0 c	–	–
2.0	–	–	0.5	0.0 c	–	–
2.0	–	–	2.0	25.0 a-c	3.5±0.5 a	19.5±10.5 ab
–	0.5	0.5	–	0.0 c	–	–
–	0.5	2.0	–	0.0 c	–	–
–	0.5	–	0.5	37.5 ab	2.3±0.9 ab	35.3±9.0 a
–	0.5	–	2.0	0.0 c	–	–
–	2.0	0.5	–	0.0 c	–	–
–	2.0	2.0	–	0.0 c	–	–
–	2.0	–	0.5	42.9 a	1.5±0.3 b	9.2±1.5 b
–	2.0	–	2.0	0.0 c	–	–

¹ Means ± standard errors in columns marked with the same letter do not differ significantly according to the HSD Tukey's test ($P \leq 0.05$)

*BA 6-benzyladenine, IAA indole-3-acetic acid, IBA indole-3-butyric acid, KIN kinetin, PGR – plant growth regulator

In Vitro Rooting and Ex Vitro Acclimatization Efficiency

Shoots produced typical roots with root hair after 18 days of culture on the rooting medium with NAA (Fig. 1d). Nonetheless, the acclimatization efficiency reached from 0.0 to 100%, depending on the cultivar and the composition of the medium used during the multiplication stage (Table 5). Plants, which did not survive the ex vitro

transfer died between the fifth and seventh days of cultivation. The survival rate of 'White Gold' plantlets was generally slightly higher than of 'Gold Heart' (64.7 and 50.0%, respectively). The acclimatization efficiency of control 'Gold Heart' microshoots (cultured on the hormone-free medium) was significantly lower (20%) compared to those produced in some PGRs-supplemented media (Table 5). As for 'White Gold', the highest survival rate (100%) was found after previous multiplication in

Table 4 Influence of culture medium composition on the concentration [mg·g⁻¹ fresh weight] of chlorophyll *a*, chlorophyll *b*, their ratio, and content of total carotenoids in shoots of in vitro-grown *L. spectabilis*

PGR* concentration [mg·L ⁻¹]				Chlorophyll <i>a</i> [mg·g ⁻¹]	Chlorophyll <i>b</i> [mg·g ⁻¹]	Chlorophyll <i>a</i> : <i>b</i> ratio	Carotenoids [mg·g ⁻¹]
IAA	IBA	BA	KIN				
Gold Heart							
–	–	–	–	0.30±0.06 ab	0.15±0.05 a-d	2.25±0.30 ab	0.076±0.008 ab
0.5	–	0.5	–	0.44±0.04 a	0.17±0.01 a-d	2.59±0.07 ab	0.105±0.007 a
0.5	–	2.0	–	0.10±0.03 bc	0.08±0.05 d	1.77±0.70 a-c	0.027±0.004 bc
0.5	–	–	0.5	0.25±0.03 a-c	0.12±0.02 b-d	2.16±0.20 a-c	0.065±0.007 ab
0.5	–	–	2.0	0.43±0.07 a	0.26±0.12 a	2.17±0.15 a-c	0.098±0.006 a
2.0	–	0.5	–	0.36±0.04 a	0.11±0.01 b-d	3.31±0.24 a	0.094±0.010 a
2.0	–	2.0	–	0.12±0.01 bc	0.09±0.02 cd	1.66±0.44 a-c	0.035±0.003 bc
2.0	–	–	0.5	0.40±0.04 a	0.16±0.01 a-d	2.61±0.41 ab	0.104±0.008 a
2.0	–	–	2.0	0.34±0.01 ab	0.22±0.01 ab	1.57±0.07 bc	0.081±0.003 ab
–	0.5	0.5	–	0.28±0.02 ab	0.16±0.03 a-d	2.10±0.54 a-c	0.077±0.009 ab
–	0.5	2.0	–	0.26±0.05 a-c	0.12±0.01 b-d	2.17±0.52 a-c	0.061±0.015 a-c
–	0.5	–	0.5	0.41±0.07 a	0.20±0.01 a-c	1.98±0.33 a-c	0.095±0.019 a
–	0.5	–	2.0	0.43±0.05 a	0.17±0.01 a-d	2.42±0.16 ab	0.113±0.008 a
–	2.0	0.5	–	0.29±0.06 ab	0.12±0.03 b-d	2.62±0.25 ab	0.079±0.014 ab
–	2.0	2.0	–	0.26±0.02 a-c	0.13±0.02 b-d	2.01±0.15 a-c	0.070±0.001 a-c
–	2.0	–	0.5	0.26±0.03 a-c	0.13±0.02 b-d	2.08±0.28 a-c	0.070±0.008 a-c
–	2.0	–	2.0	0.08±0.01 c	0.12±0.01 b-d	0.63±0.03 c	0.014±0.003 c
White Gold							
–	–	–	–	0.26±0.02 a-c	0.14±0.02 ab	1.85±0.16 ab	0.077±0.005 a-c
0.5	–	0.5	–	0.21±0.02 bc	0.11±0.01 b	1.94±0.27 ab	0.062±0.001 bc
0.5	–	2.0	–	0.16±0.03 c	0.14±0.04 ab	1.20±0.16 b	0.039±0.001 c
0.5	–	–	0.5	0.38±0.03 a	0.21±0.01 a	1.78±0.08 ab	0.105±0.005 a
0.5	–	–	2.0	0.31±0.02 a-c	0.18±0.01 ab	1.77±0.13 ab	0.081±0.005 a-c
2.0	–	0.5	–	0.30±0.02 a-c	0.19±0.01 ab	1.56±0.10 ab	0.074±0.001 a-c
2.0	–	2.0	–	0.16±0.01 c	0.13±0.01 ab	1.23±0.19 b	0.042±0.010 c
2.0	–	–	0.5	0.34±0.03 ab	0.19±0.02 ab	1.81±0.09 ab	0.081±0.007 a-c
2.0	–	–	2.0	0.36±0.03 a	0.19±0.01 ab	1.91±0.07 ab	0.093±0.008 ab
–	0.5	0.5	–	0.27±0.02 a-c	0.12±0.01 ab	2.22±0.08 ab	0.074±0.006 a-c
–	0.5	2.0	–	0.23±0.00 a-c	0.14±0.02 ab	1.70±0.16 ab	0.062±0.010 bc
–	0.5	–	0.5	0.31±0.03 a-c	0.20±0.01 ab	1.58±0.02 ab	0.078±0.007 a-c
–	0.5	–	2.0	0.35±0.03 ab	0.17±0.02 ab	2.07±0.24 ab	0.087±0.007 a-c
–	2.0	0.5	–	0.24±0.01 a-c	0.14±0.02 ab	1.80±0.33 ab	0.066±0.006 a-c
–	2.0	2.0	–	0.23±0.00 a-c	0.15±0.00 ab	1.54±0.02 ab	0.063±0.001 a-c
–	2.0	–	0.5	0.35±0.04 ab	0.16±0.05 ab	2.53±0.57 a	0.097±0.008 ab
–	2.0	–	2.0	0.39±0.05 a	0.19±0.02 ab	2.07±0.08 ab	0.096±0.012 ab

¹ Means ± standard errors in columns marked with the same letter do not differ significantly according to the HSD Tukey's test ($P \leq 0.05$)

*BA 6-benzyladenine, IAA indole-3-acetic acid, IBA indole-3-butyric acid, KIN kinetin, PGR plant growth regulator

MS medium with 2.0 mg·L⁻¹ IAA and 2.0 mg·L⁻¹ KIN or 0.5 mg·L⁻¹ IBA and 0.5 mg·L⁻¹ BA or KIN, although other experimental combinations also provided good results (50 – 80% survival rate). Plants, which survived acclimatization and were of best ('superb') quality started

to develop new vigorous leaves in the second week of ex vitro growth (Table 5). Only 'Gold Heart' plants from three experimental combinations (including the control) were of poor quality after acclimatization with numerous dead leaves visible (Fig. 1e).

Table 5 Influence of multiplication medium composition on the subsequent *ex vitro* acclimatization efficiency [%] in *L. spectabilis*

PGR* concentration [mg·L ⁻¹]				Gold Heart		White Gold	
IAA	IBA	BA	KIN	Survival [%]	Shoot quality	Survival [%]	Shoot quality
–	–	–	–	20.0 bc	+	80.0 ab	++
0.5	–	0.5	–	50.0 a-c	++	50.0 ab	++
0.5	–	2.0	–	50.0 a-c	+++	20.0 b	+++
0.5	–	–	0.5	20.0 bc	+++	20.0 b	++
0.5	–	–	2.0	80.0 ab	++	50.0 ab	++
2.0	–	0.5	–	80.0 ab	+++	80.0 ab	++
2.0	–	2.0	–	80.0 ab	+++	80.0 ab	+++
2.0	–	–	0.5	0.0 c	+	80.0 ab	++
2.0	–	–	2.0	100 a	+++	100 a	++
–	0.5	0.5	–	80.0 ab	+++	100 a	+++
–	0.5	2.0	–	50.0 a-c	+++	80.0 ab	+++
–	0.5	–	0.5	20.0 bc	+++	100 a	+++
–	0.5	–	2.0	100 a	+++	80.0 ab	+++
–	2.0	0.5	–	50.0 a-c	+++	80.0 ab	+++
–	2.0	2.0	–	0.0 c	+	20.0 b	+++
–	2.0	–	0.5	20.0 bc	+++	20.0 b	+++
–	2.0	–	2.0	50.0 a-c	++	80.0 ab	+++
Mean				50.0		64.7	

¹Means in columns marked with the same letter do not differ significantly according to the HSD Tukey's test ($P \leq 0.05$)

*BA 6-benzyladenine, IAA indole-3-acetic acid, IBA indole-3-butyric acid, KIN kinetin, PGR plant growth regulator

+- poor quality, ++ fair quality, +++ superb quality

Magnitude of Association Between the Variables Analyzed

It was found that the proliferation ratio of shoots was negatively correlated with their length ($r = -0.35$). Shoots produced in a higher number from a single explant also often contained less chlorophyll *a* and carotenoids ($r = -0.38$ and -0.40 , respectively). Similarly, increasing callogenesis efficiency and callus FW/DW had a negative impact on the content of the analyzed metabolites in the shoots (Table 6).

In contrast, there was a very strong positive association between the FW and DW in shoots ($r = 0.84$) and callus ($r = 0.97$). Experimental combinations which stimulated most frequent callus formation also positively affected its FW and DW (Table 6). Moreover, the FW and DW of callus were positively correlated with the number of shoots produced by a single explant. Longer shoots contained more chlorophyll *a* ($r = 0.61$) and carotenoids ($r = 0.64$). The concentration of carotenoids in tissues increased with the concentration of chlorophyll *a* ($r = 0.98$) and *b* ($r = 0.64$), while the content of chlorophyll *a* was positively correlated with the concentration of chlorophyll *b*. It was also found that the rooting rate, i.e., share of explants regenerating roots, was positively correlated with the root length and the number of

roots regenerated per one explant ($r = 0.80 - 0.90$). Furthermore, plants with a higher number of leaves were more likely to survive acclimatization to *ex vitro* conditions (Table 6).

Genetic Stability of In Vitro-Grown Shoots

A total of 3770 scorable bands, in the range of 153 – 2,664 bp, were detected by 10 RAPD primers (Table 7). The highest number of *loci* (12) was amplified with the R-G primer, while 4 *loci* were found with the R-A primer. Primers R-A and R-B did not detect any polymorphisms. On the other hand, 71.4% of polymorphic *loci* (within three genotypes) were reported with the R-F primer. A total of 47 polymorphic plants were detected within the RAPD analysis.

The SCoT marker system generated nearly twice as many products than RAPD; 6,915 scorable bands, in the range of 211 – 3,000 bp (Table 7). The greatest number of *loci* (21) was found with the S-E primer, while primers S-F and S-J amplified only 7 *loci*. The highest share of polymorphic *loci* was detected by the S-B primer (88.2%). Polymorphic and/or unique bands were found in 25 samples (36.8% of all plants tested).

The tested plants were grouped into two clusters, according to the RAPD analysis (Fig. 2a). Four samples with the

Table 6 Strength of association between the analyzed traits of plants, based on the Pearson correlation coefficient ($P \leq 0.05$), regardless of cultivar

Trait	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 No. of shoots	1															
2 No. of leaves	0.82	1														
3 Shoot length	−0.35	ns	1													
4 Callogenesis rate	ns	ns	ns	1												
5 Shoot FW	0.61	0.59	ns	ns	1											
6 Shoot DW	0.56	0.60	ns	ns	0.84	1										
7 Callus FW	0.52	ns	ns	0.77	ns	ns	1									
8 Callus DW	0.47	ns	ns	0.72	ns	ns	0.97	1								
9 Rooting rate	ns	ns	ns	ns	ns	ns	ns	ns	1							
10 No. of roots	ns	ns	ns	ns	ns	ns	ns	ns	0.90	1						
11 Root length	ns	ns	ns	ns	ns	ns	ns	ns	0.80	ns	1					
12 Chlorophyll <i>a</i> content	−0.38	ns	0.61	ns	ns	ns	−0.44	−0.44	ns	ns	ns	1				
13 Chlorophyll <i>b</i> content	ns	ns	ns	−0.46	ns	ns	−0.51	−0.48	ns	ns	ns	0.72	1			
14 Carotenoids content	−0.40	ns	0.64	ns	ns	ns	−0.41	−0.42	ns	ns	ns	0.98	0.64	1		
15 Chlorophyll <i>a:b</i> ratio	ns	ns	0.77	ns	ns	ns	ns	ns	ns	ns	ns	0.62	0.68	ns	1	
16 Acclimatization rate	ns	0.36	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	1

DW dry weight, FW fresh weight, ns not significant

highest genetic distance (reaching up to 2.6) were placed in a single cluster, i.e., those derived from the media supplemented with 2.0 mg·L^{−1} IBA and 2.0 mg·L^{−1} BA, 2.0 mg·L^{−1} IBA and 0.5 mg·L^{−1} BA, 2.0 mg·L^{−1} IBA and 0.5 mg·L^{−1} KIN, 0.5 mg·L^{−1} IBA and 2.0 mg·L^{−1} KIN. The remaining 64 plants were arranged in the other cluster divided into 19 sub-clusters.

UPGMA analysis of SCoT markers also showed that the tested specimens were grouped into two clusters (Fig. 2b). One genotype produced on the medium with 2.0 mg·L^{−1} IAA and 2.0 mg·L^{−1} KIN was markedly different from the others (genetic distance around 4.6). The remaining 67 plants were grouped into two sub-clusters, with a maximum genetic distance reaching 2.7.

The PCoA analysis arranged the studied samples in three groups of uneven size, regardless of the genetic marker system used (Fig. 3). However, various plants were placed in those groups for RAPD and SCoT markers, respectively.

The AMOVA analysis revealed that 64% of the total genetic variation detected with the RAPD marker system is of interindividual origin, while 36% results from variation between groups (influence of PGRs). As for the SCoT markers, the culture medium composition was the source of 45% genetic variation among the population groups tested.

Discussion

Morphogenetic Response of Explants

Despite having immense importance, *L. spectabilis* has so far received inadequate attention regarding in vitro reproduction. The obtained results are cultivar-specific, which is in agreement with other ornamental plant species (Shri et al. 2006). Interestingly, in the present study, none of the tested 16 combinations of auxins and cytokinins improved significantly the morphological parameters or multiplication ratio of bleeding heart ‘Gold Heart’ compared to the PGRs-free control. Some combinations, i.e., those containing 2.0 mg·L^{−1} IAA or IBA and 2.0 mg·L^{−1} BA or KIN, even had a deleterious effect on the shoot length, suggesting that elevating the concentration of PGRs in the culture medium is not favorable with this cultivar. Further research is needed to reveal the endogenous mechanisms of PGRs in *L. spectabilis* ‘Gold Heart’. Nonetheless, the observed here multiplication ratio (up to 5.9) is quite high and similar to that reported in *Ficus benjamina* L. ‘Natasha’ and *Heuchera × hybrida* ‘Florists Choice’ (Miler et al. 2019).

An optimal culture medium composition has been identified with bleeding heart ‘White Gold’. The MS medium supplemented with 0.5 mg·L^{−1} IBA and 2.0 mg·L^{−1} BA provided the highest number of shoots, leaves, and multiplication ratio (9.8) per one inoculated nodal segment. Auxins and cytokinins are the most essential PGRs, interacting in the regulation of cell proliferation and differentiation (Gaspar et al. 1996), which also explains the induction of

Table 7 Characteristics of molecular products obtained from the plantlets analyzed with RAPD and SCoT markers

Primer symbol	Primer sequence 5' → 3'	Total no. of bands	Size of bands [bp]		No. of loci		Total poly-morphic loci [%]	No. and (%) of plants with polymorphism	No. of genotypes detected		
			min	max	total	mono				poly	spec
RAPD											
R-A	GGG AAT TCG G	272	285	534	4	4	0	0	0 (0.0)	1	
R-B	GAC CGC TTG T	408	816	2664	6	6	0	0	0 (0.0)	1	
R-C	GGA CTG GAG T	629	169	1384	10	9	1	0	17 (25.0)	2	
R-D	GCT GCC TCA GG	189	322	1696	5	2	3	0	60.0	3	
R-E	TAC CCA GGA GCG	344	303	955	8	5	3	0	37.5	2	
R-F	CAA TCG CCG T	226	453	1233	7	2	5	0	71.4	3	
R-G	GGT GAC GCA G	545	276	1058	12	4	8	0	66.7	3	
R-H	CCC AGT CAC T	409	379	1048	7	6	0	1	14.3	2	
R-I	TGG CGT CCT T	410	153	1162	8	5	3	0	37.5	2	
R-J	AGC GTG TCT G	338	377	1597	6	4	2	0	33.3	3	
Σ (mean from a single primer)		3770 (377.0)	—	—	73 (7.3)	47 (4.7)	25 (2.5)	1 (0.1)	47 (69.1)	23	
ScdT											
S-A	CAA CAA TGG CTA CCA CCG	680	573	3000	11	9	2	0	18.2	2	
S-B	CAA CAA TGG CTA CCA CCT	943	737	2834	17	2	12	3	88.2	2	
S-C	CAA CAA TGG CTA CCA CGT	816	322	2280	13	11	1	1	15.4	2	
S-D	ACG ACA TGG CGA CCA ACG	478	536	1268	9	7	0	2	22.2	3	
S-E	ACG ACA TGG CGA CCA TCG	988	211	1721	21	13	8	0	38.1	2	
S-F	ACC ATG GCT ACC ACC GTC	476	280	1500	7	7	0	0	0 (0.0)	1	
S-G	ACC ATG GCT ACC ACC GGG	892	327	2354	15	13	2	0	13.3	2	
S-H	ACC ATG GCT ACC ACC GTG	617	520	2215	11	9	1	1	18.8	3	
S-I	CCA TGG CTA CCA CCG CCA	677	384	2191	13	6	7	0	29.0	2	
S-J	CCA TGG CTA CCA CCG CAG	348	300	813	7	5	2	0	28.6	2	
Σ (mean from a single primer)		6915 (691.5)	—	—	122 (12.2)	82 (8.2)	35 (3.5)	7 (0.7)	25 (36.8)	10	

RAPD randomly amplified polymorphic DNA, SCoT start codon target polymorphism, mono. – monomorphic, poly. polymorphic, spec. specific (unique; present in a single band profile)

Fig. 2 Dendrograms based on the estimation of genetic distance coefficient and UPGMA clustering presenting the relationships between plants from different experimental combinations, revealed by the randomly amplified polymorphic DNA (RAPD; **a** and start codon sequence repeats (SCoT; **b**) analyses. Samples representing the same band pattern as the predominant control plants are collected within a single group named ‘Monomorphic’. The scale shows a real genetic distance value. *BA* 6-benzyladenine, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, *KIN* kinetin [$\text{mg}\cdot\text{L}^{-1}$]

meristematic activity in *L. spectabilis* ‘White Gold’. The obtained here proliferation efficiency (4.0 new shoots per one explant) is higher than that in *Rosa rugosa* Thunb. ‘Tang Red’ (3.0) and identical with *R. rugosa* ‘Purple Branch’ (4.0) (Xing et al. 2010). The share of DW in shoots was on a standard level in most of the tested experimental combinations, reaching from 12.2% to even 29.0%. Moreover, there was a strong positive correlation between the FW and DW

($r=0.84$), suggesting the good quality of shoots. Only in a few PGR combinations, the share of FW fell below 10% suggesting a hyperhydration of shoots. Therefore, the described here medium, with $0.5 \text{ mg}\cdot\text{L}^{-1}$ IBA and $2.0 \text{ mg}\cdot\text{L}^{-1}$ BA, can be recommended for large-scale multiplication of *L. spectabilis* ‘White Gold’.

Callus development at the base of shoots was reported in most of the experimental combinations tested, especially in

Fig. 3 Graphs of principal coordinates analysis (PCoA) of *Lamprocapnos spectabilis* ‘White Gold’ plants produced in different culture media, based on randomly amplified polymorphic DNA (RAPD; **a** and start codon sequence repeats (SCoT; **b**) analyses. Samples representing the same band pattern as the predominant control plants are collected within a single group named ‘Monomorphic’ (part **b**). BA 6-benzyladenine, IAA indole-3-acetic acid, IBA indole-3-butyric acid, KIN kinetin [$\text{mg}\cdot\text{L}^{-1}$]

bleeding heart ‘Gold Heart’. Callogenesis efficiency in the ‘White Gold’ cultivar was higher in the presence of IBA (79.7% of explants) compared to IAA (25.0%). A combination of $0.5 \text{ mg}\cdot\text{L}^{-1}$ IBA and $2.0 \text{ mg}\cdot\text{L}^{-1}$ BA stimulated the most abundant callus formation (427.6 mg FW per explant). On the other hand, Kaviani and Kazemi (2017) reported that medium with $1.0 \text{ mg}\cdot\text{L}^{-1}$ BA and $0.3 \text{ mg}\cdot\text{L}^{-1}$ NAA yielded in the highest formation of callus (516 mg) from *Petunia* $\times$ *hybrida* hort. ex Vilm leaf culture. This underlines the great impact of the genetic factor on callogenesis.

Rooting is an integral step of most micropropagation protocols. Root system development is necessary for the survival and further growth of plants in the glasshouse. This developmental phenomenon involves the redifferentiation of predetermined cells and the initiation of several new meristematic areas (root primordia) in different tissues of stem cuttings (Yan et al. 2014). Some cultivars or plant species are capable of the simultaneous shoot and root development in vitro without an additional subculture, e.g., *Kalanchoe tubiflora* (Harvey) Hamet. (Kulus

2015b). Usually, however, an additional rooting step *in vitro* (Xing et al. 2010) or *ex vitro* (Miler and Woźny 2016) is required as cytokinins inhibit rhizogenesis. In contrast, the promotion of adventitious root formation has been considered as one of the characteristics of auxins. Similarly, in the present study, the shoots of *Lamprocapnos spectabilis* were unable to regenerate roots in most of the multiplication media tested (0 – 25% rooting efficiency and a maximum 42.9 – 62.5% in two experimental combinations). The medium supplemented solely with NAA, on the other hand, was efficient in stimulating rhizogenesis, as reported in other plant species (Yan et al. 2014). Further efforts should focus on achieving simultaneous rooting and acclimatization *ex vitro*, e.g., by soaking the shoots in the NAA solution for a predefined duration, to reduce labor and material costs.

Biochemical Stability

Production of bioactive metabolites from intact plants may be complex, due to several factors such as the impurity, heterogeneity, and inconsistency of the compounds produced. This can be improved by upgrading the *in vitro* culture systems (Raj et al. 2015). Several studies have reported that the type and concentration of PGRs affect the accumulation of metabolites in plant tissue culture systems (Grzegorzczak-Karolak et al. 2015, 2016). However, the exact mechanism of how growth regulators affect the biosynthesis of metabolites in plants is still not fully understood. None of the 16 media combinations tested in the present study enhanced significantly the content of chlorophylls or carotenoids in the shoots of *Lamprocapnos spectabilis*. Some of them even had a negative impact on those parameters compared to the control. This is surprising as cytokinins play an important role in the development and structural differentiation of plastids under *in vitro* culture (Dobránszki and Mender-Drienyovszki 2014). For example, Cioć et al. (2019) reported a higher concentration of carotenoids (0.14 – 0.16 mg·g⁻¹ FW) in the leaves of *Gerbera jamesonii* Bolus microporagated on the medium with an increased BA content. In the study with shoot culture of *Scutellaria altissima*, thidiazuron (TDZ) was most effective among other tested cytokinins in promoting secondary metabolite synthesis (Grzegorzczak-Karolak et al. 2016). Alterations in the carbon cycle, low activity of key enzymes, as well as an irregular transport mechanism and catabolism caused by *in vitro* culture stress may explain the low production of metabolites in *Lamprocapnos spectabilis* (Nitnaware et al. 2011). Especially the secretion of phenols into the medium might have caused this adverse phenomenon.

Further studies should focus on establishing an optimized protocol that can yield sustainable secondary metabolite production in *L. spectabilis*, at a minimal capital and operating

costs. The addition of ascorbic acid (or any other antioxidant) into the medium to prevent phenols secretion could be a significant improvement. According to Grzegorzczak-Karolak et al. (2016), undifferentiated callus and cell suspension cultures of *Scutellaria alpina* showed higher amounts of metabolites than the proliferating shoot cultures. Therefore, this system could also be implemented with bleeding heart, especially that in the present study, callus development had a significant impact on the content of chlorophylls and carotenoids in the shoots of both cultivars tested (Table 6).

Genetic Stability

The current study is the first to analyze the genetic stability of *Lamprocapnos spectabilis* microporagated *in vitro*. Polymorphic bands were detected in even 69.1% of plants studied. Based on the UPGMA and PCoA analyses, it can be assumed that most of the detected variation was minor and non-significant. Only four genotypes were distinctively different among the analyzed 68 plants. Callus development may be the reason for those alternations. PGRs, BA and callus-inducing auxins, in particular, are known to occasionally induce variation in microporagated plants (Miler and Zalewska 2014). Another explanation may be the free-radical mechanism. According to Cassells and Curry (2001), much of the variability expressed in microshoots may be related to oxidative stress damage caused during explant isolation and, in culture, due to an altered oxygen regime. Abundant secretion of polyphenols reported in the following study may be a symptom supporting this hypothesis. Somaclonal variation was found and sometimes even utilized in the creation of new cultivars of chrysanthemum (Miler and Zalewska 2014), sugarcane (Francischini et al. 2017), and many other plant species (Krishna et al. 2016). However, not often such a high polymorphic ratio can be found (Karp 1995; Vitamvas et al. 2019). The obtained results underline the susceptibility of bleeding heart to the action of DNA-affecting agents. Bleeding heart ‘White Gold’ can be, therefore, considered a good model organism for mutation breeding studies.

The present study demonstrated a higher discriminatory power of RAPD genetic markers (polymorphisms detected in 47 plants) compared to SCoT (polymorphisms found in 25 samples). It was also found that different primers detected variation in different plants. Various genetic markers explore different regions of the genome that have specific evolutionary patterns, which could explain the reported here differences. Another explanation is that SCoT explores only coding sequences, while RAPD also surveys noncoding sequences (Nadeem et al. 2018). Kulus et al. (2019) also confirmed the higher effectiveness of RAPDs in detecting variation in chrysanthemum, compared to inter-simple sequence repeats (ISSRs). On the other hand, in the study

by Fernández et al. (2002) and Zhnag et al. (2013), RAPDs were less successful in distinguishing genotypes compared to other marker types. It is possible that for species of the Fumariaceae family, RAPD markers are characterized by a high level of polymorphisms.

Acclimatization and Ex Vitro Growth

The quality and physiological state of micropropagated shoots are of great importance when transferring the plantlets from artificial in vitro (mixotrophic) to natural, ex vitro (autotrophic) conditions. Exogenous factors, such as culture medium components, affect the photosynthetic apparatus of the in vitro-derived plantlets (Dobránszki and Mandler-Drienyovszki 2014). Consequently, in the present study, the acclimatization efficiency reached from 0.0 to 100%, depending on the PGRs composition. The reported here mean survival rate (50.0 – 64.7%, depending on the cultivar) is comparable to that reported by Lee et al. (2004) with wild *Dicentra spectabilis* Lem. (58%). Plants that did not survive the ex vitro transfer possibly had a dysfunctional photosynthetic apparatus, e.g., deformed or constantly open stomata, enlarged intercellular spaces, lack of cuticle, etc. This is a common problem in micropropagation, resulting from the unnatural environment in vitro (Hazarika 2003). Microshoots with a higher number of leaves were more likely to survive acclimatization, probably due to a higher photosynthetic activity.

There was also a difference in the quality of plants found, depending on their origin. The presence of IBA in the multiplication medium seemed to be favorable with bleeding heart ‘White Gold’. Plants derived from all eight experimental combinations including this auxin were of superior quality after transplanting to the glasshouse compared to those produced on the IAA-supplemented medium—mostly fair quality. IBA is well known for improving the quality of micropropagated plants. It is an auxin precursor with a variety of different effects on plant growth and development; not only rooting (Frick and Strader 2017). On the other hand, there was no clear influence of the multiplication medium composition on the quality of ‘Gold Heart’ plants after acclimatization, although, most of the plants produced in the presence of PGRs were of better quality (fair or superb) compared to the control (poor quality). This also can be attributed to the impact of auxins and cytokinins on the plant expansion and photosynthetic activity (Dobránszki and Mandler-Drienyovszki 2014).

The observed here 100% survival rate in several experimental combinations, together with the superb quality of plants, are very satisfying as it is quite difficult to produce plants that can be acclimatized to the outdoor environment without any loss (Hazarika 2003). Therefore, the described

here protocols can be recommended for large-scale production of *Lamprocapnos spectabilis*.

Conclusion

This study developed a simple and practical micropropagation protocol for *Lamprocapnos spectabilis*, using common and affordable plant growth regulators. Separating the shoot multiplication and rooting steps seem necessary in bleeding heart. Culture medium fortified with 0.5 mg·L⁻¹ IBA and 2.0 mg·L⁻¹ BA is recommended for the multiplication of bleeding heart ‘White Gold’, whereas a PGRs-free medium can be utilized with the ‘Gold Heart’ cultivar (although, not directly before acclimatization). Shoots produced in those media are stable on the biochemical level. Those combinations are also securing the genetic integrity of *L. spectabilis*. Other compositions of plant growth regulators, however, can be a source of lesser or greater genetic variability. The species can be quite easily acclimatized to ex vitro conditions, after rooting in an NAA-supplemented medium, providing high-quality plant material for the ornamental and pharmaceutical markets. Moreover, due to the quite high susceptibility to somaclonal variation occurrence, *Lamprocapnos spectabilis* ‘White Gold’ might be a good model plant for mutation breeding research.

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Compliance with Ethical Standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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