



# Influence of growth regulators on the development, quality, and physiological state of *in vitro*-propagated *Lamprocapnos spectabilis* (L.) Fukuhara

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## Abstract

There is little information on the *in vitro* tissue culture systems in *Lamprocapnos spectabilis* (bleeding heart). The aim of this study was to analyze the influence of plant growth regulators (PGRs) on the development, quality, and physiological state of *in vitro*-grown bleeding heart “Gold Heart” and “White Gold”. Single-node explants were inoculated on the modified MS medium (Murashige and Skoog in *Physiol Plant* 15:473–497, 1962), fortified with different auxins, which included indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA), and picloram (PIC), along with cytokinins, which included 6-benzyladenine (BA), kinetin (KIN), and thidiazuron (TDZ) at various concentrations. The morphogenetic response of the explants was cultivar-specific. KIN was preferable for the proliferation and development of shoots in “Gold Heart.” However, none of the auxins or cytokinins improved the development of “White Gold” explants, compared with the PGR-free control medium. NAA was the most effective for stimulating rhizogenesis in both cultivars, although IAA resulted in the regeneration of the longest roots. TDZ, NAA, and PIC suppressed the development of shoots in both cultivars tested and stimulated abundant callus formation. Indirect regeneration of somatic embryos occurred on the NAA- and PIC-fortified media. In particular, the latter media stimulated regeneration of the highest number of somatic embryos per nodal segment. Composition of the culture medium also affected the levels of primary and secondary metabolites in shoots and callus of *L. spectabilis*. IAA (at 1.0 mg L<sup>-1</sup>) stimulated the synthesis of chlorophyll *a* and carotenoids in the “Gold Heart,” while BA and KIN (at 0.5 mg L<sup>-1</sup>) had a negative impact on the concentration of chlorophyll *b* in the shoots of this cultivar. None of the PGRs increased the level of the pigments in the shoots of bleeding heart “White Gold.” The concentration of chlorophylls and carotenoids in the callus of both cultivars tested was significantly lower compared with the shoots; however, callus was abundant in flavanols.

**Keywords** Bleeding heart · Callogenesis · Caulogenesis · Rhizogenesis · Somatic embryo

## Introduction

*Lamprocapnos spectabilis* (L.) Fukuhara or bleeding heart is a popular herbaceous perennial, native to northeast Asia (Roberts *et al.* 1995). It is a temperate, long-day shade plant species (Hodges 2012). Based on DNA sequencing and pollen and seed coat anatomy observations, the species was reclassified in 1997 in the small botanical family –

Fumariaceae (previously Papaveraceae) (Lidén *et al.* 1997). Bleeding heart has arching stems (racemes), with more than ten pendent red, pink, or white cordate flowers in two planes of symmetry. The flowers have a high ornamental value on the floristic market and in gardening (Hodges 2012). At the base of each outer flower petal is a spur, which holds nectar from the glands. The nectar is abundant in nitrogenous secondary metabolites, such as berberine, cheilantifoline, coptisine, scoulerine, and other isoquinolines, which are valuable for medical use (Iwasa and Kim 1997; McNulty *et al.* 2007).

*Lamprocapnos spectabilis* is propagated mainly vegetatively by cuttings (Hodges 2012). The economy of this method, however, is reduced by the limited number of offspring produced. Moreover, it is affected by environmental conditions, such as temperature, humidity, and sunlight during the relatively long horticultural timeframe. It is also time-

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consuming to maintain and requires significant expenditure. *In vitro* reproduction of plants overcomes those problems and has become an important segment of the economy (Kulus 2015). Due to the enormous potential in horticulture, pharmacology, and cosmetology, the study of bleeding heart in the laboratory is justified and needed.

*In vitro* tissue culture is currently the most basic tool used in both basic and applied plant studies and for commercial purposes. It is based on totipotency, which is the unique ability of plants to recover an entire organism from just a single non-zygotic cell (or even individual protoplasts). The *in vitro* technologies are applied to an increasing number of plant species; however, information on tissue culture in bleeding heart is limited to the induction of somatic embryogenesis by 2,4-dichlorophenoxyacetic acid (2,4-D) (Lee and Lee 2003; Lee *et al.* 2004) and should be investigated.

The efficiency of a tissue culture system depends on numerous elements that require careful consideration. They can be divided into two main groups, which include (1) physical factors, such as light and temperature conditions in the growth room or explant parameters, and (2) chemical factors, such as the type and concentration of plant growth regulators (PGRs), osmotic and solidifying agents, and pH of the culture medium. In the study by Miler *et al.* (2019), optimal light parameters were set for *in vitro* cultivation of *L. spectabilis* using fluorescent tubes and wide-spectrum light emitting diodes (LEDs). The study showed that fluorescent tubes were superior to LEDs in terms of propagation ratio, shoot length, and leaf number. However, the role of PGRs in the development of bleeding heart explants, or any other Fumariaceae species cultured *in vitro*, is still not well understood.

The effects of natural and synthetic plant growth regulators are rarely specific in their influence on growth and development *in vitro* and can vary greatly with culture conditions or the genotype (Gaspar *et al.* 1996). Apart from regulating cell growth and differentiation, PGRs are among the most significant factors that affect the metabolite production in plants. *In vitro*-grown plant material provides the advantage of a continuous and reliable source of valuable pharmaceuticals, because yield is normally low in field-grown plants and influenced by variations in environmental conditions (Mohammad *et al.* 2014). Considering the medicinal potential of the species, it would be beneficial to know how various PGRs affect the levels of primary and secondary metabolites in *in vitro*-cultivated bleeding heart, as no such information is currently available.

The aim of this study was to provide for the first time some basic knowledge of the influence of various common and affordable plant growth regulators (auxins and cytokinins), on the development, quality, and physiological state of two of the most popular cultivars of *Lamprocapnos spectabilis* (“Gold Heart” and “White Gold”), cultivated *in vitro*.

## Materials and Methods

### Culture medium and physical conditions in the growth room

The modified Murashige and Skoog (MS) medium (Murashige and Skoog 1962), with increased by half concentration of calcium II chloride ( $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ ) and iron sulfate ( $\text{FeSO}_4$ ), supplemented with 3% (w/v) sucrose, and solidified with 0.8% (w/v) agar (Biocorp, Warsaw, Poland), was used in the experiment. The pH was adjusted to 5.8 (with 0.1-M HCl and 0.1-M NaOH; Chempur, Piekary Śląskie, Poland), after adding all media components (Chemia, Bydgoszcz, Poland), prior to autoclaving at 105 kPa and 121 °C for 20 min (autoclave SMS ASVE, Góra Kalwaria, Poland). The medium (40 mL) was poured into 350-mL glass jars and sealed with plastic caps (produced by a local manufacturer).

The cultures were kept in the growth room at  $24 \text{ °C} \pm 1 \text{ °C}$ , under 16-h photoperiod conditions and photosynthetic photon flux density of approximately  $33.8 \mu\text{mol m}^{-2} \cdot \text{s}^{-1}$ , which was provided by standard cool daylight TLD 54/36 W fluorescent tubes, with a color temperature of 6200 K (Koninklijke Philips Electronics N.V., Eindhoven, Netherlands).

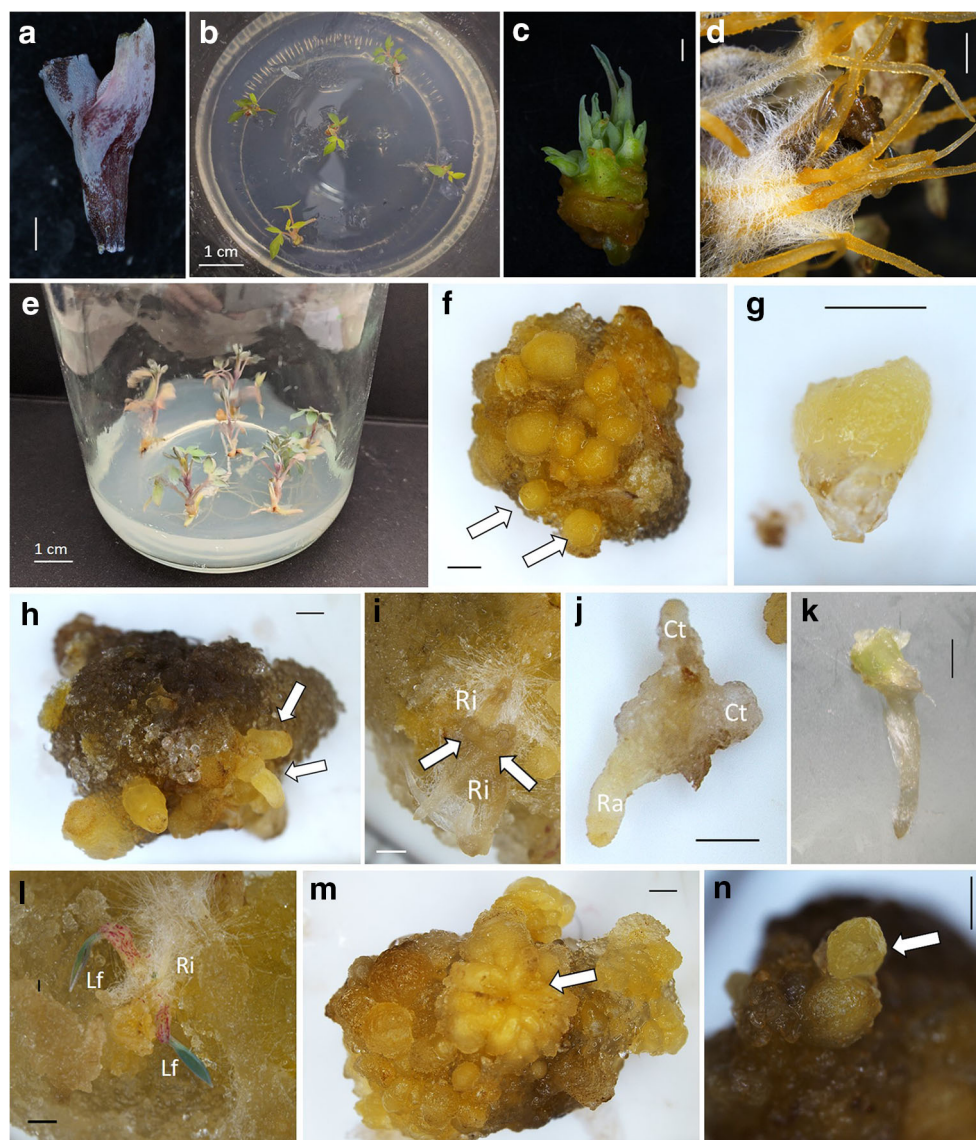
**Biological material and multiplication of plants** *In vitro*-derived plantlets of *Lamprocapnos spectabilis* (L.) Fukuhara “Gold Heart” and “White Gold” were used as the source of explants. Axenic cultures of the two cultivars were obtained from the international commercial plant tissue culture laboratory Vitroflora in Łochowo, Poland.

Donor plants, approximately 10 to 12 cm long, were cloned using the single-node method in the modified MS medium (described earlier), devoid of plant growth regulators, to produce the appropriate amount of plant material. For this purpose, shoots were cut into nodal segments and subcultured on a fresh medium with six explants per jar, every 16 wk. for 8 mo. One inoculated explant produced one shoot, from which approximately eight secondary explants could be dissected.

### Effect of various growth regulators on the morphogenetic response of explants

The 16-wk-old shoots were cut into single-node segments, 5 to 10 mm long (Fig. 1a), without leaves and inoculated vertically in the modified MS medium. Various types of auxins, which included indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA), picloram (PIC), and cytokinins, which included 6-benzyladenine (BA), kinetin (KIN), and thidiazuron (TDZ), were added into the medium at the concentration of 0.5 or 1.0 mg L<sup>-1</sup>. All PGRs were provided by Sigma-Aldrich®, St. Louis, MO. A PGR-free control was also included. The *in vitro* cultures were cultivated for 8 wk.

**Biometrical analysis of plants** After a single propagation cycle, the number, length [mm], and fresh weight [mg] of the shoots were measured. The rooting efficiency [%], number, and



**Figure 1.** *In vitro* development of *Lamprocapnos spectabilis* (L.) Fukuhara “Gold Heart” (GH) and “White Gold” (WG), on the modified MS medium (Murashige and Skoog 1962), fortified with various plant growth regulators. *a* Nodal segments used in the experiment; *b* early development of nodal explants (GH, MS + 1.0-mg L<sup>-1</sup> KIN); *c* proliferation of axillary shoots (GH, MS + 0.5-mg L<sup>-1</sup> KIN); *d* regeneration of roots with root hair (WG, MS + 0.5-mg L<sup>-1</sup> NAA); *e* well-developed microshoots (GH, MS + 1.0-mg L<sup>-1</sup> IAA); and *f–n* indirect development of somatic embryos (indicated with arrows) (*f* globular stage (WG, MS + 1.0-mg L<sup>-1</sup> PIC); *g* heart stage (WG, MS + 0.5-mg L<sup>-1</sup> PIC); *h* torpedo

stage (GH, MS + 0.5-mg L<sup>-1</sup> PIC); *i* somatic embryos regenerating roots (Ri) (GH, MS + 1.0-mg L<sup>-1</sup> NAA); *j* germinated embryo with cotyledons (Ct) and radicle (Ra) (GH, MS + 1.0-mg L<sup>-1</sup> PIC); *k* well-developed embryo (GH, MS + 1.0-mg L<sup>-1</sup> PIC); *l* conversion into complete plantlet with roots (Ri) and leaves (Lf) (GH, MS + 1.0-mg L<sup>-1</sup> PIC); *m* multi-embryo (GH, MS + 1.0-mg L<sup>-1</sup> PIC); and *n* secondary embryogenesis (WG, MS + 1.0-mg L<sup>-1</sup> PIC). Photos taken after 3 wk. (*b–c*) and 8 wk. (*d–n*) in culture. Bar = 1 mm, unless otherwise stated. IAA indole-3-acetic acid; KIN kinetin; NAA 1-naphthaleneacetic acid; PIC picloram.

length [mm] of roots were also included. Moreover, the share of explants regenerating callus [%], its diameter [mm], morphology, fresh weight [mg], and embryogenetic potential was estimated. The number and developmental stage of somatic embryos (if present), regenerated by a single explant, were evaluated.

Photographical documentation was performed with the use of a COOLPIX L820 camera and SMZ1500 stereoscopic microscope (Nikon, Tokyo, Japan).

**Biochemical array** Spectrophotometric analysis of *in vitro*-grown plant pigments, extracted from at least five 100-mg fresh shoot or callus samples (if no shoots were available), from every experimental combination was performed. The extraction of chlorophylls, carotenoids, and flavanols was performed with the method described by Lichtenthaler (1987), with 100% acetone (Chemia, Bydgoszcz, Poland). The spectrophotometric analysis of extracts was performed using a spectrophotometer (SmartSpec Plus™, Bio-Rad

Laboratories, Hercules, CA, and UV-VIS 1601-PC SHIMADZU, Kyoto, Japan).

Absorption maxima were defined for pigment-specific wavelengths ( $\lambda_{\max}$ ) of 330 nm for flavanols, at 470 nm for carotenoids, and at 645 and 662 nm for chlorophyll *a* and *b*, respectively. The level of chlorophylls and carotenoids per g of fresh matter was calculated with the algebraic method following Lichtenthaler and Buschmann (2001).

**Statistical analysis** The single-factor experiment was repeated four times for two cultivars, independently. One jar with five explants was considered as a single repetition.

For the data expressed as a percentage, the Freeman-Tukey transformation was used. After the appropriate normality transformation, the results (completely randomized design) were statistically analyzed with one-way ANOVA, and the comparisons of means were made with HSD Tukey's multiple comparison test ( $P \leq 0.05$ ) using Statistica 12.0 and ANALVAR-5.2-FR tools. Tables with results provide real numerical data, while alphabet letters point to homogenous groups, which follow the statistical calculations based on transformed data.

## Results

The development of callus on the NAA-, TDZ-, and PIC-supplemented media began in the second week of culture. For the other PGRs, callus formation started in the third or fourth week, followed by the control (only in "Gold Heart"). The development of shoots and roots (if present), on the PGR-supplemented media, was quite simultaneous and evident in the third week of culture (Fig. 1*b*). For the PGR-free control, roots started to regenerate only after shoots were developed with at least a few leaves.

**Development of shoots and callogenesis efficiency** The highest number of bleeding heart "Gold Heart" shoots produced by a single explant (3.3), with the greatest number of leaves (22.9), was found on the medium fortified with 0.5 mg L<sup>-1</sup> KIN (Table 1; Fig. 1*c*). Shoots produced on this medium were also longer (30 mm per explant), compared with the control (13.4 mm). The greatest fresh weight of shoots per explant (159.0 to 324.8 mg) was reported with 0.5-mg L<sup>-1</sup> KIN and 1.0-mg L<sup>-1</sup> BA (Table 1).

With the "White Gold" cultivar, none of the auxins or cytokinins added into the medium had a positive effect on the proliferation of shoots, their length, fresh weight, or number of leaves compared with the PGR-free control (Table 1).

TDZ, NAA, and PIC suppressed the formation of shoots in both cultivars tested and stimulated abundant callus formation in all of the explants (Table 1). Callus was also formed at the base of shoots in the presence of BA (83.3–100% explants) and KIN (37.5 to 81.2%), although in much lower levels.

**Rooting efficiency** Application of PGRs was necessary to stimulate rhizogenesis, as only 8.3% of "Gold Heart" explants and 25% in "White Gold" explants produced roots spontaneously in the control medium (Table 2). Those 25% "White Gold" shoots, however, regenerated the highest mean number of roots (6.5). The highest rooting efficiency (87.5 to 100% explants) was reported in the medium supplemented with NAA, regardless of the concentration (Fig. 1*d*). Root formation was less frequent in the presence of IAA (33.3 to 75.0% explants; Fig. 1*e*), while the application of cytokinins (except for KIN in "White Gold") and PIC inhibited rooting. Similarly, the explants of both cultivars regenerated the highest number of roots in the NAA-supplemented medium (3.8 to 6.3 per explant); however, the longest roots (12.6 to 20.1 mm) were found in the medium with 1.0-mg L<sup>-1</sup> IAA (Table 2).

**Callus morphology and somatic embryogenesis** The morphology and structure of callus depended on the type of PGR used, although not on the concentration, or the cultivar (Table 3). NAA, especially at higher concentrations, stimulated the formation of callus with the highest diameter (18.8 to 23.8 mm per explant) and fresh weight (1703.5 to 1816.0 mg).

Callus that formed on the medium with TDZ had no embryogenic or organogenic potential, regardless of the cultivar (Table 3). It was firm and yellow-green in color. There were also no somatic embryos that regenerated in bleeding heart "White Gold" on the NAA-supplemented medium. Sole application of this auxin did stimulate embryogenesis in the "Gold Heart" cultivar (Table 3). A greater share of embryogenic callus (63.3% of all inoculated explants) was found if a higher (1.0 mg L<sup>-1</sup>) concentration of NAA was used, compared with 0.5 mg L<sup>-1</sup> (31.7%). The highest share of nodal segments that formed embryogenic callus (66.7–100%) and the highest mean number of somatic embryos for each inoculated explant (2.9 to 4.1) were reported in the presence of PIC in both cultivars tested, regardless of the concentration.

All somatic embryos had an indirect origin. They were bipolar (starting from the heart stage) and had a typical shape and glossy appearance (Fig. 1*f–n*). The embryos could be easily isolated from the explant; they had no vascular connections with the mother tissue. Typical developmental stages of somatic embryos, which included globular (Fig. 1*f*), heart (Fig. 1*g*), and torpedo (Fig. 1*h*), were observed. Some of the embryos already formed radicles, roots and cotyledons, or leaves (Fig. 1*i–l*). Multi-embryos (several somatic embryos connected in an inseparable clump) (Fig. 1*m*) and secondary embryos (embryos regenerating from another somatic embryo) were also found (Fig. 1*n*). Most of the somatic embryos were yellow, and only some were green.

Globular somatic embryos were usually dominant (40 to 100% of all embryos), regardless of the medium composition or cultivar (Fig. 2). Modified MS medium with 1.0-mg L<sup>-1</sup> NAA stimulated the development of embryos at torpedo stage in bleeding heart "Gold Heart" (60% of embryos). Only a few

**Table 1** Influence of culture medium composition on the number of *Lamprocapnos spectabilis* (L.) Fukuhara shoots and leaves formed from a single-node explant, shoot length [mm], fresh weight [mg], and the share [%] of explants forming callus

Medium composition		No. of shoots	No. of leaves	Shoot length [mm]	Shoot fresh weight [mg]	Callus [%]
PGR* type	Concentration [mg L <sup>-1</sup> ]					
<b>Gold Heart</b>						
Control	–	1.0 ± 0.2 cf <sup>1</sup>	4.2 ± 1.0 de	13.4 ± 1.9 b	107.6 ± 27.7 b	33.3 ab
BA	0.5	1.8 ± 0.3 bc	12.1 ± 1.0 bc	22.6 ± 1.3 ab	140.4 ± 24.0 b	83.3 a
	1.0	1.5 ± 0.3 bd	11.2 ± 2.0 bc	25.4 ± 4.8 ab	324.8 ± 92.2 a	100 a
KIN	0.5	3.3 ± 0.5 a	22.9 ± 2.2 a	30.0 ± 3.5 a	159.0 ± 36.4 ab	58.3 ab
	1.0	2.5 ± 0.5 ab	14.6 ± 2.7 b	25.9 ± 2.0 ab	124.6 ± 26.7 b	75.0 ab
TDZ	0.5	0.4 ± 0.2 df	0.4 ± 0.4 e	n.a.	n.a.	100 a
	1.0	0.5 ± 0.2 df	0.4 ± 0.2 e	n.a.	n.a.	100 a
IAA	0.5	1.2 ± 0.1 ce	6.8 ± 1.0 cd	20.8 ± 3.0 ab	81.0 ± 28.2 b	0.0 b
	1.0	1.2 ± 0.1 ce	8.2 ± 0.6 cd	24.6 ± 2.8 ab	72.8 ± 13.0 b	25.0 ab
NAA	0.5	0.2 ± 0.1 ef	0.2 ± 0.2 e	n.a.	n.a.	100 a
	1.0	0.0 f	0.0 e	n.a.	n.a.	100 a
PIC	0.5	0.0 f	0.0 e	n.a.	n.a.	100 a
	1.0	0.0 f	0.0 e	n.a.	n.a.	100 a
<b>White Gold</b>						
Control	–	1.8 ± 0.2 a	8.1 ± 0.4 ab	20.6 ± 1.1 a	116.2 ± 13.5 ab	0.0 c
BA	0.5	1.2 ± 0.1 ab	8.4 ± 1.3 ab	14.9 ± 0.8 b	110.7 ± 16.3 ab	100 a
	1.0	1.3 ± 0.2 ab	7.6 ± 0.8 ab	15.3 ± 1.2 ab	171.8 ± 33.4 a	93.8 a
KIN	0.5	1.4 ± 0.2 a	7.4 ± 0.8 ab	13.6 ± 1.2 b	71.9 ± 11.2 b	37.5 b
	1.0	1.7 ± 0.2 a	10.4 ± 1.2 a	20.8 ± 1.3 a	136.3 ± 15.9 ab	81.2 a
TDZ	0.5	0.7 ± 0.2 bc	0.7 ± 0.2 c	n.a.	n.a.	100 a
	1.0	0.3 ± 0.2 c	0.3 ± 0.2 c	n.a.	n.a.	100 a
IAA	0.5	1.1 ± 0.1 ab	6.4 ± 0.5 b	14.9 ± 0.8 b	55.7 ± 10.2 b	6.2 bc
	1.0	1.1 ± 0.1 ab	7.6 ± 0.8 ab	18.4 ± 1.9 ab	96.4 ± 23.3 ab	0.0 c
NAA	0.5	0.2 ± 0.1 c	0.4 ± 0.3 c	n.a.	n.a.	100 a
	1.0	0.1 ± 0.1 c	0.2 ± 0.1 c	n.a.	n.a.	100 a
PIC	0.5	0.0 c	0.0 c	n.a.	n.a.	100 a
	1.0	0.0 c	0.0 c	n.a.	n.a.	100 a

<sup>1</sup> Means in columns ± standard errors 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; KIN kinetin; n.a. not available due to tiny size of shoots; NAA 1-naphthaleneacetic acid; PGR plant growth regulator; PIC picloram; TDZ thidiazuron

somatic embryos at maturity stage (developing root and leaves) were found in “Gold Heart” on the medium containing 1.0-mg L<sup>-1</sup> PIC (Fig. 2).

**Biochemical array** There was a significant influence of the culture medium composition on the level of pigments in *L. spectabilis* (Table 4). The highest concentrations of chlorophyll *a* and carotenoids in bleeding heart “Gold Heart” were found in shoots cultured on the medium with 1.0-mg L<sup>-1</sup> IAA. BA (regardless of concentration) and KIN (at 0.5 mg L<sup>-1</sup>) had a negative impact on the level of chlorophyll *b* in the shoots of this cultivar (Table 4). The chlorophyll *a:b* ratio in callus usually reached a value < 1.0 (except for the callus formed on the

medium with 1.0-mg L<sup>-1</sup> PIC). However, a higher share of chlorophyll *a* to *b* could be found in shoots.

For bleeding heart “White Gold,” BA (especially at higher concentrations) had a negative impact on the synthesis of chlorophylls and carotenoids in shoots. In addition, KIN at 1.0 mg L<sup>-1</sup> decreased by half the level of chlorophyll *b* in shoots (0.12 mg g<sup>-1</sup> fresh weight) compared with the control (0.24 mg g<sup>-1</sup>). None of the PGRs enhanced the biosynthesis of the analyzed pigments in this cultivar compared with the control (Table 4). Similarly to “Gold Heart,” the chlorophyll *a:b* ratio in “White Gold” was also usually higher in shoots (> 1.0) compared with callus (< 1.0), except for the calluses formed on the media with 1.0-mg L<sup>-1</sup> NAA and 1.0-mg L<sup>-1</sup> PIC.

**Table 2.** Influence of culture medium composition on the share [%] of *Lamprocapnos spectabilis* (L.) Fukuhara explants forming roots, number, and length [mm] of roots for each inoculated or rooted explant

Medium composition		Rooting [%]	No. of roots per inoculated explant	No. of roots per rooted explant	Root length per inoculated explant [mm]	Root length per rooted explant [mm]
PGR* type	Concentration [mg L <sup>-1</sup> ]					
Gold Heart						
Control	—	8.3 d	0.2±0.2 bc	2.0±0.0 bc	0.8±0.8 bc	10.0±0.0 ab
BA	0.5	0.0 d	0.0 c	—	0.0 c	—
	1.0	0.0 d	0.0 c	—	0.0 c	—
KIN	0.5	0.0 d	0.0 c	—	0.0 c	—
	1.0	0.0 d	0.0 c	—	0.0 c	—
TDZ	0.5	0.0 d	0.0 c	—	0.0 c	—
	1.0	0.0 d	0.0 c	—	0.0 c	—
IAA	0.5	33.3 c	1.4±0.7 b	4.3±0.9 ac	11.8±7.5 ab	35.5±8.6 a
	1.0	75.0 b	1.4±0.4 b	1.9±0.5 c	20.1±5.5 a	26.8±5.8 a
NAA	0.5	100 a	5.5±0.6 a	5.5±0.6 ab	4.9±0.7 bc	4.9±0.7 b
	1.0	100 a	6.3±0.6 a	6.3±0.6 a	4.7±0.5 bc	4.7±0.5 b
PIC	0.5	0.0 d	0.0 c	—	0.0 c	—
	1.0	0.0 d	0.0 c	—	0.0 c	—
White Gold						
Control	—	25.0 cd	1.6±0.8 bc	6.5±1.9 a	3.3±1.5 ab	13.0±0.7 ab
BA	0.5	0.0 d	0.0 c	—	0.0 b	—
	1.0	0.0 d	0.0 c	—	0.0 b	—
KIN	0.5	62.5 ac	2.3±0.7 ac	3.6±0.9 ab	5.9±1.7 ab	9.5±2.0 b
	1.0	43.8 c	1.6±0.6 bc	3.6±0.8 ab	7.9±3.2 ab	18.0±5.3 ab
TDZ	0.5	0.0 d	0.0 c	—	0.0 b	—
	1.0	0.0 d	0.0 c	—	0.0 b	—
IAA	0.5	50.0 bc	1.6±1.0 bc	3.3±1.8 ab	8.8±2.4 ab	15.6±2.4 ab
	1.0	42.5 bc	0.9±0.4 c	2.1±0.8 b	12.6±6.0 a	28.7±11.3 a
NAA	0.5	100 a	4.3±0.6 a	4.3±0.6 ab	8.5±1.9 ab	8.5±1.9 b
	1.0	87.5 ab	3.8±0.5 ab	4.3±0.5 ab	3.9±0.7 ab	4.5±0.7 b
PIC	0.5	0.0 d	0.0 c	—	0.0 b	—
	1.0	0.0 d	0.0 c	—	0.0 b	—

<sup>1</sup> Means in columns ± standard errors 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; KIN kinetin; NAA 1-naphthaleneacetic acid; PGR plant growth regulator; PIC picloram; TDZ thidiazuron

The absolute concentration of chlorophyll *a*, chlorophyll *b*, and carotenoids in the callus of both cultivars tested was usually significantly lower compared with the shoots (even 100 to 1000 times, as in the case of carotenoids in callus developed on the NAA-supplemented medium). However, the callus was abundant in flavanols (Fig. 3).

## Discussion

The present research confirmed the applicability of nodal segments in the *in vitro* studies with bleeding heart, as all of them demonstrated some type of morphogenetic response, such as caulogenesis, rhizogenesis, callogenesis, and/or embryogenesis. The suitability of this explant type was also reported by We *et al.*

(2015), with *Sauropus androgynus* (L.) Merr. In their research, nodal segments produced a comparable number of adventitious shoots (7.8) as internodes (5.5) and leaf segments (10.0), in the same culture conditions. In addition, the share of responding explants was similar in most experimental combinations tested (83.3 to 96.7%). In bleeding heart, the morphogenetic and physiological activity of explants was PGR-dependent.

**Activation of axillary meristem and biometrical parameters of shoots** In the present study, the effect of various PGRs on the *in vitro* development and quality of two bleeding heart cultivars was tested. Auxins and cytokinins are among the main regulators of *in vitro* growth and development. Their effect depends on the biological material, their concentration, conjugation with other compounds, and the interaction with endogenous and artificial

**Table 3.** Influence of culture medium composition on the callus morphology, structure, diameter [mm], and fresh weight [mg]; share of *Lamprocapnos spectabilis* (L.) Fukuhara explants forming embryogenic callus [%]; and mean and maximal number of somatic embryos regenerated by a single explant

Medium composition		Dominant callus color and structure <sup>1</sup>	Callus diameter [mm]	Callus fresh weight [mg]	Embryogenic callus [%]	Mean no. of embryos	Max no. of embryos
PGR type*	Concentration [mg L <sup>-1</sup> ]						
<b>Gold Heart</b>							
TDZ	0.5	yellow-green	14.9 ± 0.7 bc	497.4 ± 51.3 c	0.0 c	0.0 b	—
	1.0	firm	14.6 ± 0.9 bc	474.0 ± 60.9 c	0.0 c	0.0 b	—
NAA	0.5	creamy	18.4 ± 1.6 b	1007.8 ± 141.1 b	31.7 bc	1.1 ± 0.5 b	4
	1.0	medium-hard	23.8 ± 1.8 a	1816.0 ± 219.1 a	63.3 ab	2.2 ± 0.6 ab	7
PIC	0.5	yellow-gray	13.0 ± 0.9 c	470.3 ± 79.6 c	100 a	3.7 ± 0.9 a	12
	1.0	soft	10.9 ± 0.9 c	207.4 ± 37.6 c	100 a	4.1 ± 0.9 a	9
<b>White Gold</b>							
TDZ	0.5	yellow-green	12.0 ± 0.6 b	262.6 ± 24.6 c	0.0 b	0.0 b	—
	1.0	firm	9.4 ± 0.5 b	158.8 ± 17.6 c	0.0 b	0.0 b	—
NAA	0.5	creamy	18.8 ± 1.3 a	943.9 ± 180.3 b	0.0 b	0.0 b	—
	1.0	medium-hard	22.7 ± 1.5 a	1703.5 ± 285.6 a	0.0 b	0.0 b	—
PIC	0.5	yellow-gray	11.1 ± 0.7 b	298.8 ± 32.7 bc	66.7 a	2.9 ± 1.0 a	10
	1.0	soft	8.1 ± 0.4 b	142.1 ± 16.9 c	93.8 a	3.2 ± 0.6 a	11

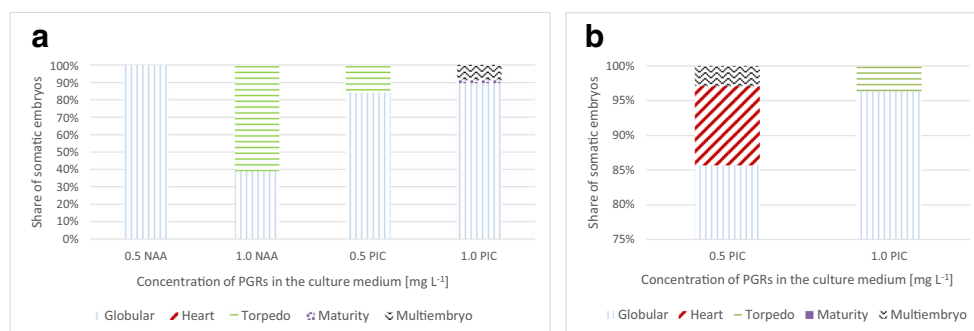
<sup>1</sup> Means in columns ± standard errors marked with the same letter do not differ significantly according to the HSD Tukey's test ( $P \leq 0.05$ )

\* BA 6-benzyladenine; IAA indole-3-acetic acid; KIN kinetin; NAA 1-naphthaleneacetic acid; PGR plant growth regulator; PIC picloram; TDZ thidiazuron

growth regulators or other physical factors in the growth room. For example, in the study by Pahnekolayi *et al.* (2015), the cytokinin BA alone was the most effective for stimulating shoot proliferation in *Rosa canina* L. nodal segments, compared with several other combinations of BA, gibberellic acid (GA<sub>3</sub>), and NAA. NAA auxin (1.0 mg L<sup>-1</sup>) was preferable for stimulation of adventitious shoot regeneration from leaf explants of *Ajanía pacífica* “Silver and Gold,” compared with BA or TDZ (Tymoszuk and Antkowiak 2018). For the encapsulated nodal segments of chrysanthemum, the effect of various cytokinins (CKs) was cultivar-specific (Kulus and Zalewska 2014). In the present study, addition of 0.5-mg L<sup>-1</sup> KIN to the culture medium was optimal for bleeding heart “Gold Heart” shoot proliferation, shoot length, and number of leaves. However, a higher fresh weight (FW) of shoots was reported when 1.0-mg L<sup>-1</sup> BA was applied. FW is an important parameter to describe the leaf

biomass and area. In addition, FW reflects the physiological functions of leaves associated with photosynthesis and respiration (Huang *et al.* 2019). The present study confirmed that cytokinins are more effective than auxins for stimulating the activation of lateral buds and the formation of multiple axillary shoots of high quality in *L. spectabilis* “Gold Heart.” It should be emphasized that none of the PGRs tested improved the biometric parameters of shoots of bleeding heart “White Gold.” This cultivar may have other requirements for optimal growth *in vitro*.

**Rhizogenesis** Auxins stimulate the expression of numerous genes in the plant. Disturbance of the homeostasis can have great effects on root growth and development (Overvoorde *et al.* 2010). The present study confirmed that similar to other plant species (Simon and Petrášek 2011), bleeding heart rooting is also induced by IAA and NAA. Spontaneous rooting on a PGR-free

**Figure 2.** Influence of culture medium composition on the maturation of somatic embryos in *Lamprocapnos spectabilis* (L.) Fukuhara “Gold Heart” (a) and “White Gold” (b) after 8 wk. in culture. NAA 1-naphthaleneacetic acid; PIC picloram; PGRs plant growth regulators.

**Table 4.** Influence of culture medium composition on the concentration of chlorophyll *a*, chlorophyll *b*, and total carotenoid levels in the recovered shoots and callus\*\* of *Lamprocapnos spectabilis* (L.) Fukuhara “Gold Heart” and “White Gold” [mg g<sup>-1</sup> fresh tissue]

Medium composition		Chlorophyll <i>a</i> [mg g <sup>-1</sup> ]	Chlorophyll <i>b</i> [mg g <sup>-1</sup> ]	Chlorophyll <i>a:b</i> ratio	Carotenoids [mg g <sup>-1</sup> ]
PGR* type	Concentration [mg L <sup>-1</sup> ]				
<b>Gold Heart</b>					
Control	—	0.42 ± 0.05 b <sup>1</sup>	0.26 ± 0.01 a	1.59 ± 0.15 bc	0.11 ± 0.01 bc
BA	0.5	0.39 ± 0.03 bc	0.16 ± 0.01 bc	2.51 ± 0.14 ab	0.10 ± 0.01 bc
	1.0	0.30 ± 0.03 c	0.09 ± 0.01 cd	3.55 ± 0.52 a	0.09 ± 0.01 c
KIN	0.5	0.41 ± 0.02 b	0.15 ± 0.01 bc	2.80 ± 0.15 ab	0.10 ± 0.001 bc
	1.0	0.47 ± 0.03 b	0.25 ± 0.02 a	1.89 ± 0.09 bc	0.12 ± 0.01 bc
TDZ	0.5	0.04 ± 0.00 d**	0.06 ± 0.01 d**	0.71 ± 0.11 c	0.01 ± 0.001 d**
	1.0	0.02 ± 0.01 d**	0.05 ± 0.01 d**	0.48 ± 0.07 c	0.01 ± 0.001 d**
IAA	0.5	0.49 ± 0.04 ab	0.20 ± 0.02 ab	2.46 ± 0.05 ab	0.13 ± 0.01 ab
	1.0	0.62 ± 0.06 a	0.26 ± 0.04 a	2.49 ± 0.22 ab	0.16 ± 0.01 a
NAA	0.5	0.03 ± 0.01 d**	0.05 ± 0.01 d**	0.55 ± 0.08 c	0.001 ± 0.001 d**
	1.0	0.02 ± 0.01 d**	0.03 ± 0.01 d**	0.73 ± 0.23 c	0.002 ± 0.001 d**
PIC	0.5	0.03 ± 0.01 d**	0.05 ± 0.01 d**	0.52 ± 0.04 c	0.01 ± 0.001 d**
	1.0	0.03 ± 0.02 d**	0.04 ± 0.03 d**	1.24 ± 0.49 bc	0.01 ± 0.001 d**
<b>White Gold</b>					
Control	—	0.41 ± 0.05 ab	0.24 ± 0.05 a	1.93 ± 0.17 ac	0.11 ± 0.01 ab
BA	0.5	0.26 ± 0.03 cd	0.09 ± 0.02 bc	2.98 ± 0.41 a	0.09 ± 0.01 b
	1.0	0.14 ± 0.03 de	0.06 ± 0.01 bc	2.35 ± 0.59 ac	0.05 ± 0.01 c
KIN	0.5	0.35 ± 0.04 bc	0.17 ± 0.02 ac	2.10 ± 0.13 ac	0.09 ± 0.01 b
	1.0	0.36 ± 0.02 bc	0.12 ± 0.01 bc	3.12 ± 0.26 a	0.10 ± 0.001 b
TDZ	0.5	0.06 ± 0.03 e**	0.10 ± 0.05 bc**	0.63 ± 0.06 bc	0.01 ± 0.001 d**
	1.0	0.04 ± 0.01 e**	0.05 ± 0.01 bc**	0.67 ± 0.04 bc	0.01 ± 0.001 d**
IAA	0.5	0.41 ± 0.03 ab	0.19 ± 0.02 ab	2.20 ± 0.25 ac	0.12 ± 0.01 ab
	1.0	0.53 ± 0.04 a	0.20 ± 0.02 ab	2.77 ± 0.32 ab	0.13 ± 0.001 a
NAA	0.5	0.01 ± 0.001 e**	0.03 ± 0.02 c**	0.40 ± 0.04 c	0.0001 ± 0.00 d**
	1.0	0.02 ± 0.001 e**	0.04 ± 0.02 c**	1.31 ± 0.73 ac	0.001 ± 0.000 d**
PIC	0.5	0.05 ± 0.02 e**	0.08 ± 0.03 bc**	0.60 ± 0.04 bc	0.02 ± 0.001 d**
	1.0	0.01 ± 0.01 e**	0.03 ± 0.01 c**	3.09 ± 1.43 a	0.01 ± 0.001 d**

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

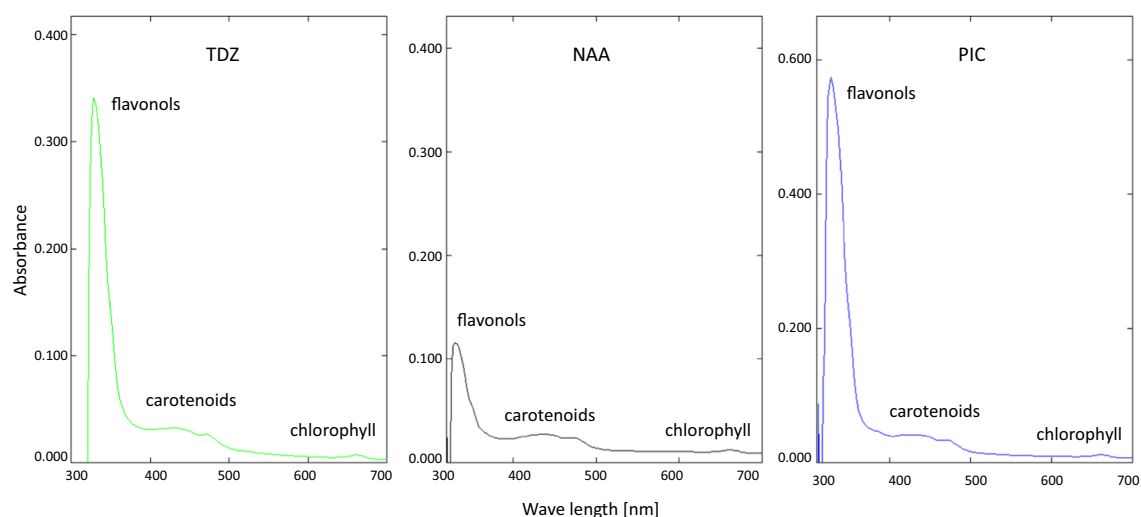
\* BA 6-benzyladenine; IAA indole-3-acetic acid; KIN kinetin; NAA 1-naphthaleneacetic acid; PGR plant growth regulator; PIC picloram; TDZ thidiazuron

\*\* Concentration of pigments in callus

medium is much less effective (8.3–25.0% rooting rate). BA also inhibited rhizogenesis in both cultivars tested. Interestingly, KIN had a positive influence on root development in the “White Gold” cultivar (62.5% rooting rate). Similar results were reported with four cultivars of chrysanthemum (Kulus and Zalewska 2014). This phenomenon could be explained by the model proposed by Jones *et al.* (2010), in which cytokinins function as positive regulators of auxin biosynthesis.

Perhaps using a medium supplemented with both cytokinin and auxin could be beneficial to stimulate efficient root formation and shoot elongation of *L. spectabilis*, without the need for subsequent subcultures, as shown with *Rauvolfia tetraphylla* (Alatar and Faisal 2012).

**Callogenesis** Auxins promote cell growth expansion, initiation of cell division, and organization of meristems, which give rise to either unorganized tissue (callus) or defined organs (mainly roots) (Kou *et al.* 2013). In *L. spectabilis*, except for the naturally occurring IAA, auxins were predominant in callogenesis stimulation in both cultivars tested at low concentrations (0.5 mg L<sup>-1</sup>). Similar to *Solanum tuberosum* L. (Kawochar *et al.* 2017), NAA was essential for dynamic callus formation in bleeding heart. CKs were less effective for promoting this process, except for TDZ, which is in agreement with the studies by Thiruvengadam *et al.* (2010) and Zhang *et al.* (2011). The varied callus induction ability between plant growth regulators can be explained by different “sensitivity” of tissues to specific hormones. This



**Figure 3.** Absorbance curves of carotenoid, chlorophyll, and flavonol extracts in callus of *Lamprocapnos spectabilis* (L.) Fukuhara “White Gold” produced on the modified Murashige and Skoog medium

supplemented with  $0.5\text{-mg L}^{-1}$  thidiazuron (TDZ), 1-naphthaleneacetic acid (NAA), or picloram (PIC) after 8 wk. in culture.

“sensitivity” and the effect of PGR change and depend on the developmental stage (degree of totipotency) and age of the tissue culture (Gaspar *et al.* 1996). Additional studies could focus on the induction of callus tissue with various morphology and structures in bleeding heart and the analysis of their chemical composition, as callus cultures can be a good source of pharmaceuticals (Mohammad *et al.* 2014).

**Somatic embryogenesis in bleeding heart** Apart from external agents, such as the culture medium composition or physical parameters in the growth room, the success or failure of the embryogenetic response is determined by several internal factors, such as age and type of explant, the level of endogenous PGRs, and also the genotype of the mother plant. Some species are considered “difficult” for inducing somatic embryogenesis, because the process can be repressed by the secreted *in vitro* metabolites, such as phenolic compounds, as reported in the *Coffea* genus (Nic-Can *et al.* 2015). Lema-Rumińska and Niedojadło (2010) obtained only 0.02 to 0.26 embryos per shoot explant in *Copiapoa tenuissima* Ritt f. *monstruosa*. It is also difficult to induce somatic embryogenesis in some woody plants (Guan *et al.* 2016). However, it is highly efficient with *Citrullus colocynthis* (L.) Schrad to generate true type clones (Ramakrishna and Shastree 2016) and in large-scale and synchronic production of *Anthurium andraeanum* Lind. seedlings (Wang *et al.* 2019). These results suggest that *Lamprocapnos spectabilis* may be classified as a species with high embryogenetic potential, as somatic embryos were easily induced (up to 12 per 1-cm-long explant), in the medium supplemented with a low concentration (even  $0.5\text{ mg L}^{-1}$ ) of PIC and in the “Gold Heart” cultivar with common and affordable NAA. In comparison, Constantin *et al.* (2015) used  $20\text{-mg L}^{-1}$  NAA for embryogenic callus induction from leaf explants of *Elaeis guineensis* Jacq. var. *Tenera*. Auxins, usually 2,4-D, are the most

broadly documented factor associated with the formation of embryogenic callus and pre-embryogenic masses (PEMs). The use of exogenous auxins induces the expression of various genes, which are primarily transcription factors (TFs), which modify the genetic program of the somatic cell and regulate the explants metabolism, which result in the activation of its embryogenic potential (Méndez-Hernández *et al.* 2019). Complementary with the present study, PIC ( $2.0$  to  $4.0\text{ mg L}^{-1}$ ) was also most effective for stimulating the proliferation of embryogenic callus in two carnation cultivars (Karami and Kordestani 2007). It is possible that this auxin might be a primary signal that initiates embryo formation in bleeding heart.

The somatic embryos obtained were morphologically similar to those described in numerous other plant species (Joshi and Kumar 2013). However, not all developmental stages typically observed in somatic embryogenesis could be found in all experimental combinations. For example, embryos at the heart stage were found only in the medium with  $0.5\text{-mg L}^{-1}$  PIC. This may be related to the well-known issue of asynchronous development of embryos and the influence of various types and concentrations of PGRs on this phenomenon (Shen *et al.* 2018).

**Effect of PGRs on the level of metabolites in plants** Analysis of biochemical compounds is important, particularly for plants of medicinal use such as bleeding heart (Kalia *et al.* 2016). The biosynthetic activity of the plant can be affected by the type and concentration of PGRs used. Lucchesini *et al.* (2009) found that the level of valuable substances, such as caftaric, chlorogenic, and cichoric acids, in *in vitro*-grown *Echinacea angustifolia* DC. plants, was higher than in those grown *in vivo*, probably due to the presence of growth regulators in the culture medium. In the present study, the level of carotenoids and chlorophylls in bleeding heart was also affected by the medium composition.

In addition to stimulating the proliferation of cells, cytokinins are also involved in the biosynthesis of chlorophyll and development of plastids. Consequently, it is often observed that somatic embryos formed on media supplemented only with auxins have a creamy/yellow color (Portillo *et al.* 2007), which is in agreement with this study. Cioć *et al.* (2019) found that the level of chlorophyll *a* and carotenoids in the shoots of *in vitro*-produced *Gerbera jamesonii* Bolus increased with increasing concentrations of BA. Similarly, an exogenous application of CKs decreased chlorophyll degradation in different plant species grown *in vivo* (Dobrąński and Mendler-Drienyovszki 2014). In the present study however, shoots of the two cultivars of bleeding heart recovered on the IAA-supplemented medium, often had higher chlorophyll and carotenoid concentrations compared with those grown in media with BA or KIN. This may suggest that the chloroplasts of shoots grown in CK-supplemented media were dysfunctional, as their proper development is necessary for the normal response to CKs found by Kulaeva *et al.* (2002). Moreover, degradation of chlorophylls and carotenoids may be a symptom of premature senescence of the plants (Karatas *et al.* 2010). Therefore, the use of structural analogues of BA, such as 6-benzyl-adenine riboside (BAR) or meta-topolin (TOP), might be recommended for bleeding heart. Similar to these results, IAA had a stimulatory effect on the biosynthesis of chlorophylls and carotenoids in *Wolffia arrhiza* (L.) Wimm, unlike its analogues (NAA and phenylacetic acid (PAA)) (Czerpak *et al.* 2002). Those results confirmed that the effect of PGRs strongly depends on the biological material.

It is worth mentioning that *in vitro* cultivation of “Gold Heart” plants in a medium with 1.0-mg L<sup>-1</sup> IAA increased the concentration of the tested compounds, by approximately 50%, compared with the control. Therefore, *in vitro* culture could be used for sustainable production of any metabolite of interest in bleeding heart. Those results could be important for the pharmaceutical industry for the overproduction of constituents with medicinal use and should be explored in the future.

## Conclusions

The present study demonstrated that the morphogenetic response of nodal explants of bleeding heart is cultivar- and PGR-specific. The addition of 0.5-mg L<sup>-1</sup> KIN to the modified MS medium improved the biometrical parameters of axillary shoots in *L. spectabilis* “Gold Heart” (their number, length, and number of leaves). This effect, however, is not observed with “White Gold.” NAA and IAA are preferable for rhizogenesis induction and root elongation, respectively, in both cultivars tested. It was also quite easy to induce indirect somatic embryogenesis in both cultivars studied in the presence of 0.5 to 1.0-mg L<sup>-1</sup> PIC or NAA (in “Gold Heart” only). Composition of the culture medium significantly affected the synthesis of primary and secondary

metabolites in the tissues of bleeding heart. The auxin IAA, at 1.0 mg L<sup>-1</sup>, increased the production of chlorophyll *a* and carotenoids in the shoots by 50% compared with the PGR-free control. These results serve as a first step to optimize culture media for a variety of purposes, such as for *in vitro* propagation, conservation, metabolite extraction, or plant breeding.

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## Compliance with ethical standards

**Conflict of interest** The author declares that there are no conflicts of interest.

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