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PLANT DEVELOPMENT/REGENERATION

Goji berry (*Lycium barbarum* L.) *in vitro* multiplication improved by light-emitting diodes (LEDs) and 6-benzylaminopurine

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Abstract

In Brazil, *Lycium barbarum* L. (goji berry) is an agronomically valuable, imported, commercialized plant. The present study evaluated the effects of cytokinins and light sources on optimization of *in vitro* multiplication of goji berry by axillary bud proliferation. Nodal explants were excised from plants 90 d after *in vitro* germination and cultured on Murashige and Skoog (MS) medium containing 30 g L^{-1} sucrose; 7 g L^{-1} agar-agar; and 0, 2, 4, 8, or 16 μM 6-benzylaminopurine (BAP), 6-furfurylaminopurine (KIN), or thidiazuron (TDZ). After 60 days, regenerated lateral buds and shoot lengths were evaluated. The regenerated shoots were inoculated in MS supplemented with 0.0, 2.5, 5.0, 10.0, or 20.0 μM BAP under white fluorescent lamps or mixed treatment of red/blue (RB) light-emitting diode (LED) lamps. After 60 days *in vitro* cultivation, the number of formed shoots, leaf number, and shoot length were determined. Subsequently, the shoots were separated and acclimatized in a greenhouse for 30 d and the percentage of survival was evaluated. High regeneration and shoot numbers during lateral bud regeneration occurred with MS medium plus 4 μM BAP. The estimated optimum concentration of 11.56 μM BAP under RB LED lamp resulted in a high shoot number (8.15), with more average leaves per plant (9.2) at 5 μM BAP, and longer shoots (12.34 cm) at 12.42 μM BAP. Acclimatization was successful with 100% survival of *ex vitro* plants. An efficient *in vitro* multiplication protocol of an economically important species, goji berry, was developed to facilitate large-scale commercialization of this species.

Keywords Goji berry (*Lycium barbarum* L.) micropropagation · Cytokinins · Light sources · Medicinal species · Solanaceae

The fruits of *Lycium barbarum* L., commonly known as goji berries, are referred to as 'super-fruits,' due to their beneficial constituents (Xin *et al.* 2013; Fratianni *et al.* 2018), and are highly valued for their medicinal properties (Lam *et al.* 2016). Some of the prominent therapeutic effects reported for goji berry fruit extracts are antioxidant potential and free radical scavenging (Wu *et al.* 2004), aging prevention (Li *et al.* 2007), antitumor potential (Kulczyński and Gramza-Michałowska 2016), reduction of blood lipids (Pai *et al.* 2013), and reduction of glycemia (Ming *et al.* 2009).

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Despite its proven medicinal importance and growing commercial interest, goji berry cultivation remains problematic, because germination is non-uniform, making it difficult to grow new plants with good quality and productivity (Tudor *et al.* 2017). Micropropagation, a common plant tissue culture technique, allows for fast, asexual propagation in a controlled environment (Trigiano and Gray 2016; Al-Khayri and Naik 2017). Likewise, this approach offers the prospect of reducing costs while producing high-quality planting material in countries currently importing goji berry; the five biggest importers in 2014 included the Netherlands, the USA, Hong Kong, Germany, and Brazil.

Published papers indicate protocols exist for *in vitro* goji berry regeneration from stem fragments (Fira *et al.* 2016), protoplasts (Ratushnyak *et al.* 1989), apical meristems, leaves, roots, calluses, and somatic embryos (Hu *et al.* 2008; Osman *et al.* 2013a; Osman *et al.* 2013b; Dănăilă-Guidea *et al.* 2015). However, there are reports of plantlet hyperhydricity after *in vitro* regeneration, which increases the difficulty and

reduces the chances of successful acclimatization and consequent multiplication (Youlong *et al.* 2008). Among the types of explants used in *in vitro* propagation, lateral buds can be a viable alternative to maximize production of elite clones, because they have meristematic cells that can be obtained in great amounts from a single plant.

Additionally, previously published reports aiming to optimize goji berry micropropagation only regarded the use of 6-benzylaminopurine (BAP) alone, or in combination with 1-naphthaleneacetic acid (NAA), as plant growth regulators (Dănăilă-Guidea *et al.* 2015; Fira *et al.* 2016). As small changes in plant growth regulator concentrations often have pleiotropic effects, and this hormonal balance determines further developmental pathways of a plant cell (Khan *et al.* 2015), finding the best concentration of different plant growth regulators is necessary to optimize *in vitro* propagation.

The light source is another relevant factor for *in vitro* cultivation, since it has great influence on plantlet growth, regeneration, and subsequent acclimatization (Farhadi *et al.* 2017). Light-emitting diodes (LEDs), especially red and blue LEDs, have been proven to promote better plant growth, improved morphogenic response, and increased biosynthesis of volatile compounds, compared with plants grown under white fluorescent lights (Andrade *et al.* 2015; Manivannan *et al.* 2017). The main property of LEDs that produces these increased physiological responses in plants is due to the precise spectrum of light emitted, which can be fitted to the exact requirements of each species and developmental process (Cioć *et al.* 2018). However, studies using LEDs as the main light source during the *in vitro* cultivation of goji berry have not yet been performed.

Given the above, this present study aimed to evaluate, firstly, the effects of different phytoregulators on bud regeneration and, secondly, the effects of two light sources in order to optimize the *in vitro* multiplication of goji berry lateral buds.

Goji berry seeds were obtained from Planeta Sementes (Valença, Brazil), collected separately from five elite productive plants with different genotypes. The fruits were selected and allowed to soak in distilled water for 8 h, to facilitate removal of the seeds. A total of 200 seeds were surface-sterilized for 30 s in 200 mL of 70% (*v/v*) ethanol, followed by immersion for 10 min in 200 mL of 0.1% (*w/v*) mercuric chloride ($HgCl_2$) (Merck KGaA, Darmstadt, Germany) solution, according to Hu *et al.* (2006). Decontaminated seeds were washed thrice with autoclaved distilled water. Thereafter, seeds were inoculated on an induction medium of MS (Murashige and Skoog 1962) basal salts, 30 g L^{-1} sucrose, and 7 g L^{-1} agar-agar (Sigma-Aldrich®, St. Louis, MO). The medium pH was adjusted to 5.7 with 2 N NaOH and HCl prior to autoclaving at 121°C and 111.5 kPa, for 20 min. The seeds (25 per plate) were cultured in 90 × 15 mm sterile polystyrene petri dishes (J. Prolab®, São Paulo, Brazil) containing 25 mL aliquots of medium, which were sealed with vinyl polycloret film (Rolopac®, São Paulo, Brazil). During this phase, cultures were maintained under culture room conditions at 25 ± 2°C, with a 16-h photoperiod, and 36 μmol m^{-2} s^{-1} irradiance provided by two 20-W fluorescent tubes (Osram, Barueri, Brazil).

After approximately 90 days of culture, 1 cm long nodal explants with a single node were obtained from the *in vitro*–germinated plants and inoculated onto culture medium composed of MS basal salts, 30 g L^{-1} sucrose, 7 g L^{-1} agar-agar, and supplemented with 0.0, 2.0, 4.0, 8.0, or 16.0 μM 6-benzylaminopurine (BAP), 6-furfurylaminopurine (KIN), or thidiazuron (TDZ). After inoculation, the explants were again kept in a growth room at 25 ± 2°C, with a 16-h photoperiod, and 36 μmol m^{-2} s^{-1} light intensity, provided by two 20 W fluorescent tubes (Osram).

Each experimental unit (from germinated plants) consisted of 3 nodal explants per 250-mL glass flask (SP Medica®, São Paulo, Brazil); each flask represented one replication, with a total of 15 replicates per treatment, arranged completely at random. After 60 days of *in vitro* cultivation, the percentage of regenerated buds and the shoot lengths were determined.

To evaluate *in vitro* multiplication, individualized shoots from regenerated *in vitro* lateral buds after 90 d of cultivation were used, which had been previously cultured for 15 d on MS culture medium without the presence of growth regulators. After this period, the shoots were standardized at 4.0 cm in length, having 3 nodes, and were inoculated into culture medium containing MS basal salts, 30 g L^{-1} sucrose, and gelled with 7 g L^{-1} agar-agar (Sigma-Aldrich®). The treatments consisted of a bifactorial combination of five BAP concentrations (0.0, 2.5, 5.0, 10.0, or 20.0 μM) and two types of light sources: (i) 20-W white fluorescent lamp with a white (400–700 nm) light at 36 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) and (ii) mixed treatment of red LED (peak at 630 nm) and blue LED (peak at 460 nm) (RB LED) with 86 μmol m^{-2} s^{-1} PPFD. Both (i) and (ii) light sources were obtained from Osram, Brazil. The spectral distributions in relative energy of the white fluorescent (Fig. 1*a*) and RB LED (Fig. 1*b*) lamps were determined with a Model 1400 LI-COR® light quantum meter (LI-COR® Inc., Lincoln, NE). After inoculation, the explants were again kept at a temperature of 25 ± 2°C, with a 16-h photoperiod.

Each experimental unit consisted of 3 shoots (4.0 cm in length with 3 nodes) per glass flask (250 mL, SP Medica®, São Paulo, Brazil), each flask represented one replication, with a total of 30 replicates per treatment, arranged completely at random. The variables evaluated after 60 d of *in vitro* culture were shoot number per explant, leaf number per shoot, and shoot length.

Regenerated shoots were separated and roots treated with 20 mg L^{-1} Rootone® (Bayer Crop Science, Durham, NC) (98% total concentration (TC); 1-naphthaleneacetic acid (NAA)), according to Tonkha and Dzyazko (2014).

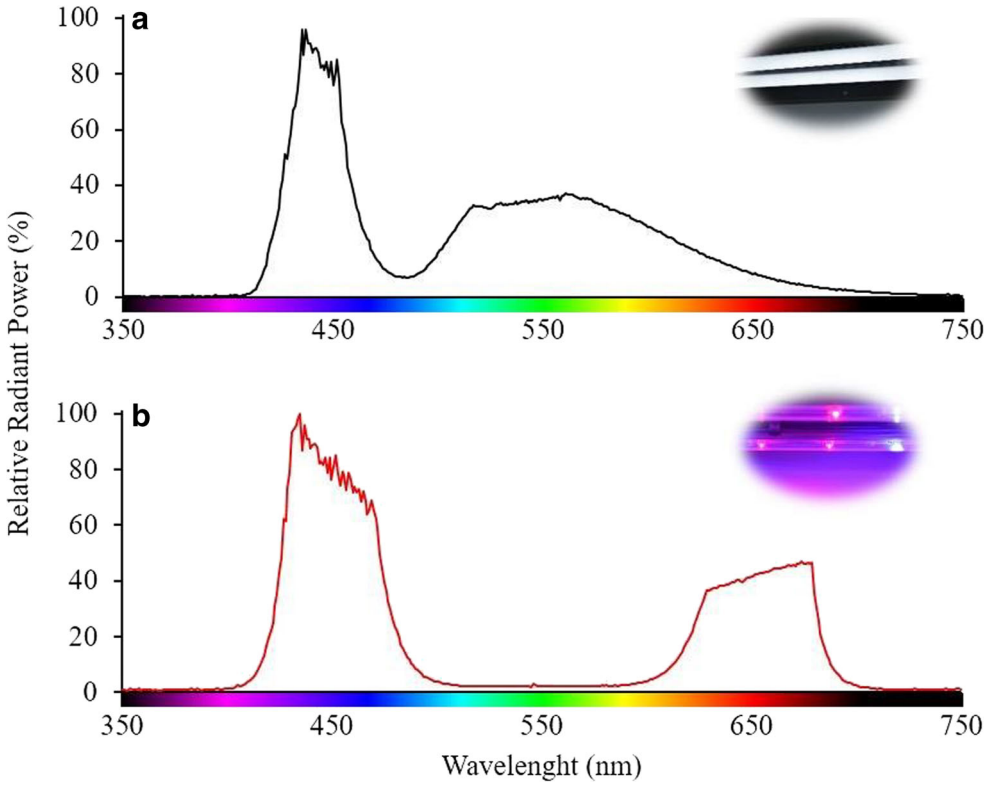

Figure 1. Spectral distributions in *relative radiant power* of (*a*) the White fluorescent lights, (shown in *insert*), and, (*b*) red and blue light–emitting diode lamps (shown in *insert*).

Subsequently, plantlets were transferred to polyethylene pots (500 mL) filled with 500 g of a commercial substrate (Tropstrato®; Genfértil, Campinas, Brazil). The pots were covered with clear plastic bags (NZB PACK, Socorro, Brazil) to maintain high relative humidity at the beginning of acclimatization. The plantlets were grown under a controlled temperature of 27 ± 2°C in a greenhouse and watered daily with 50 mL of distilled water. After 21 days of acclimatization, the plastic bag was opened and then gradually removed, and the percentage of plantlet survival was evaluated.

The experimental design was completely randomized for all experiments and all experimental procedures were repeated three times. Data were submitted to analysis of variance (ANOVA) using the statistical software SISVAR 4.3 (System Analysis of Variance for Balanced Data; Lavras,

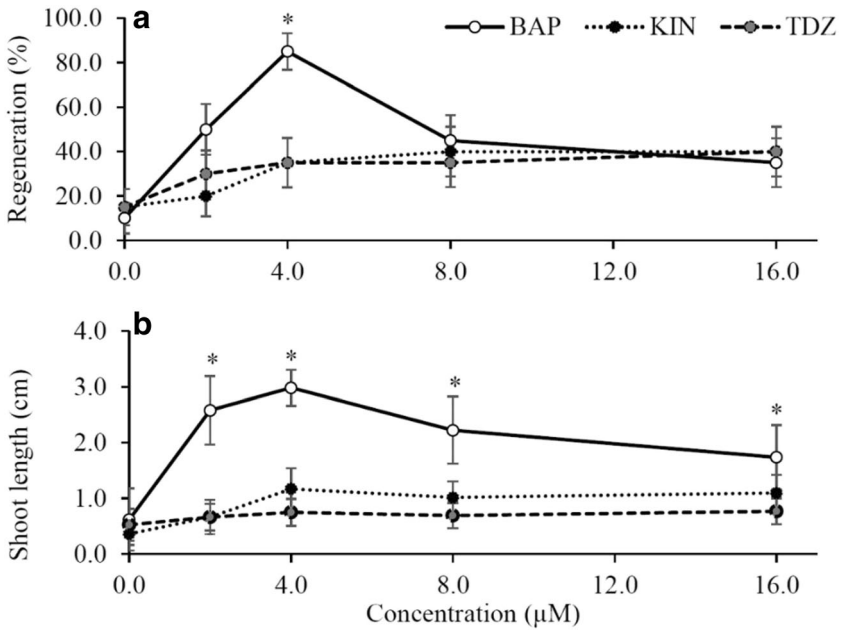

Figure 2. Effect of 0, 2, 4, 8, or 16 μM 6-benzylaminopurine (*BAP*), 6-furfurylaminopurine (*KIN*), and thidiazuron (*TDZ*) on *Lycium barbarum* L. (*a*) lateral bud regeneration rate and (*b*) shoot length. *Asterisk* indicates a significant difference compared with the control (0 μM), according to the Tukey test at 5% significance.

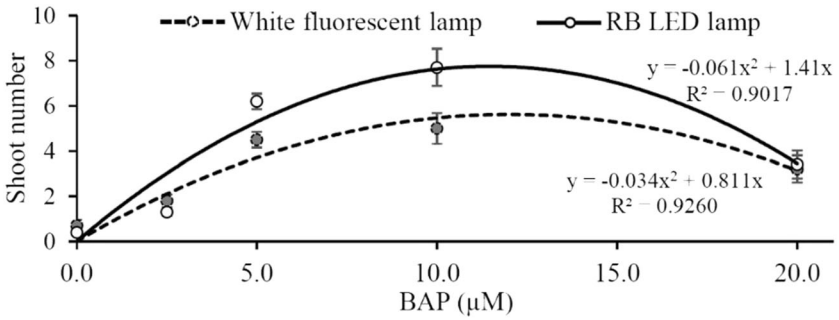

Figure 3. A polynomial regression model describing shoot number as a function of 6-benzylaminopurine (*BAP*) concentration under *White fluorescent lamp* (*filled circles*, *dashed line*) or red and blue light–emitting diode (*RB LED*) *lamp* (*open circles*, *solid line*) during the *in vitro* multiplication of *Lycium barbarum* L. (goji berry).

Brazil), as described by Ferreira (2014). According to the results of the ANOVA, data were compared by Tukey's test ($P < 0.05$), or by polynomial regression ($P < 0.05$).

A significant effect on the regeneration of goji berry lateral buds was observed in MS basal salts supplemented with 4.0 μM BAP, which yielded more than 80% regeneration response (Fig. 2*A*). In contrast, regeneration response of explants grown with higher concentrations of BAP, KIN, or TDZ leveled off around 40% (Fig. 2*a*). Additionally, the greatest length of regenerated lateral buds was observed with BAP treatment, where lateral buds were up to 3 times longer than with KIN or TDZ (Fig. 2*b*).

Since it was demonstrated that BAP resulted in higher regeneration percentage and shoot length of lateral buds, this treatment was selected for the multiplication step. Thus, it was possible to fit a polynomial regression model that describes the number of new shoots according to the BAP concentration in different light sources. Using the equation, it was estimated that, at a concentration of 11.56 μM BAP, a maximum of 8.15 shoots per explant were observed under RB LED illumination (Fig. 3), compared with 11.93 μM BAP with the maximum 4.84 shoots per explant regenerated under white fluorescent lights (Fig. 3). A visual comparison of shoot growth per explant of tissues grown with 10 μM BAP under either white fluorescent (Fig. 6*a*) or RB LED (Fig. 6*b*) illumination further supported this finding.

The leaf number of new shoots was also evaluated, considering the two types of light sources; however, this response did not follow a polynomial regression model and each treatment was individually evaluated. Thus, it was determined that all BAP concentrations tested in both light sources resulted in a greater leaf number, when compared with the control (0.0 μM BAP; Fig. 4), although the highest leaf number occurred at 5 μM BAP (9.2 leaves) under RB LED lamp (Fig. 4). Finally, shoot length was evaluated and a polynomial regression model was fitted to each light source (Fig. 5). By applying the equation, it was possible to determine that the BAP concentration of 12.42 μM yielded the highest shoot length (12.34 cm) under a RB LED lamp, representing a 64% shoot length increase found with RB LEDs, compared with 13.97 μM BAP yielding 7.53-cm-long shoots under white fluorescent lamps (Fig. 5).

After this multiplication period, 100% of the individualized plantlets (established under either light condition) that were transferred to greenhouse conditions for acclimatization presented leaves and roots (Fig. 6*c*).

This study provided results about the regeneration of lateral buds in response to different types and concentrations of cytokinins and optimized *in vitro* shoot multiplication rate with various BAP concentrations under RB LEDs in goji berry.

The results presented here were in agreement with previous findings that BAP-supplemented medium proved to be

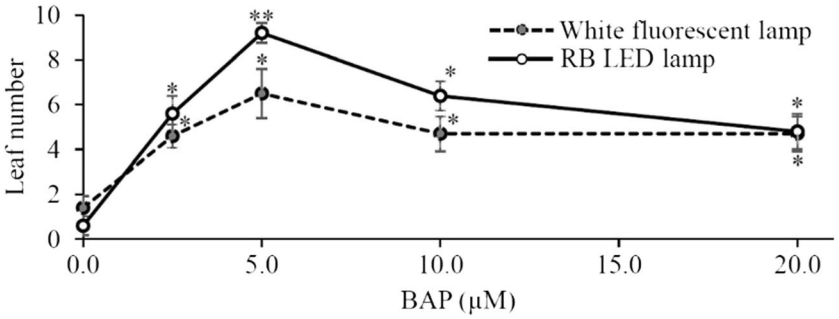

Figure 4. Effect of *White fluorescent lamp*, *(filled circles*, *dashed line)* and red and blue light–emitting diode (*RB LED*) *lamp,* (*open circles*, *solid line)*) on leaf number during the *in vitro* multiplication of *Lycium barbarum* L. (goji berry) at different 6-benzylaminopurine (*BAP*) concentrations. *Asterisk* indicates a significant difference compared to the control (0 μM) and *double asterisks* indicate a significant difference between the other concentrations, according to the Tukey test at 5% significance.

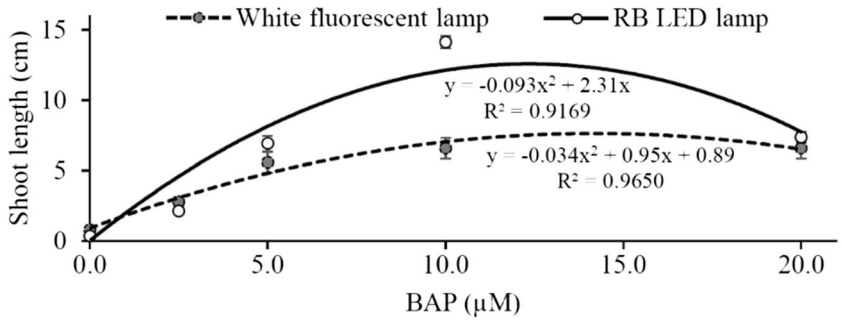

Figure 5. Polynomial regression model that describes shoot length as a function of 6-benzylaminopurine (*BAP*) concentration under *White fluorescent lamp* (*filled circles*, *dashed line*) and red and blue light–emitting diode (*RB LED*) *lamp* (*open circles*, *solid line*) during *Lycium barbarum* L. (goji berry) multiplication.

effective for the *in vitro* multiplication of goji berry, as the explants exhibited high proliferation rates and well-developed plantlets (Fira *et al.* 2016). Nevertheless, evaluating the additional cytokinins TDZ or KIN was necessary for assurance of protocol optimization. White fluorescent lamps are often used to provide controlled radiation to plants grown *in vitro*; however, this kind of lamp emits light deficient in red wavelengths, which can be detrimental to the growth of plants (Al-Mayahi 2016). Therefore, care must be taken to provide a suitable spectral balance for culture growth (Al-Mayahi 2016). Light-emitting diodes have numerous additional advantages over conventional lamps, including low-power requirement, determined spectral composition, small size and mass, durability, wavelength specificity, and cool emitting surfaces (Gupta and Jatothu 2013). As a result, LEDs have emerged as a novel and efficient light source for promoting plant growth, reducing manufacturing costs, and expanding the scope for their application in commercial micropropagation (Gupta and Jatothu 2013).

The effects of LED system as a monochromatic, monochromatic and dichromatic, source on various aspects of plant health have been reported for several plant species grown under *in vitro* conditions, such as, *Vaccinium corymbosum* L. micropropagation (Hung *et al.* 2016), *Vitis vinifera* L. (grape) transcription levels (Li *et al.* 2017), *Peucedanum japonicum* Thunb. (Chen *et al.* 2016) and *Saccharum* spp. (sugarcane) somatic embryogenesis (Ferreira *et al.* 2017), *Lupinus angustifolius* L. early flowering (Croser *et al.* 2016), and *Lippia alba* (Mill.) N.E.Br. ex Britton & P. Wilson essential oil profile (Batista *et al.* 2016).

The spectral quality of lights is expressed by the relative intensity and quantity of different wavelengths emitted by a light source and perceived by photoreceptors within a plant (Lin *et al.* 2013). In the current study, an increase of 68% in shoot number, 41% in leaf number, and 64% in shoot length was observed under RB LED lamps, compared with white fluorescent lamps. This showed that the intensity and quantity of the different wavelengths emitted by the RB LED lamp were better adjusted to promote goji berry explant growth than those of the white fluorescent lamp, and thus lead to the increased development of new shoots.

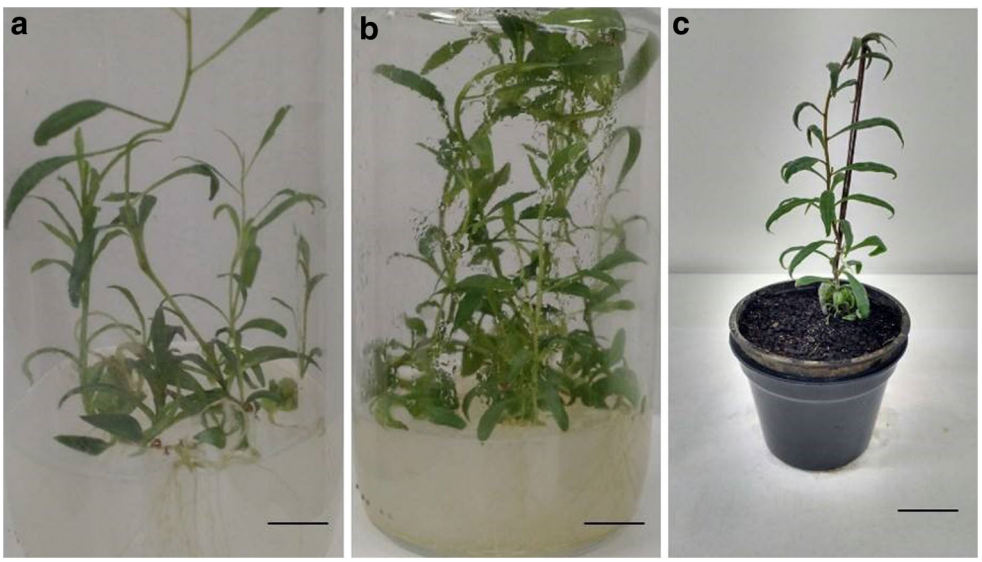

Figure 6. Visual appearance of *Lycium barbarum* L. (goji berry) shoots obtained from lateral buds cultured in MS (Murashige and Skoog 1962) culture medium basal salts plus 10 μM 6-benzylaminopurine grown under (*a*) white fluorescent lamps and (*b*) under red and blue light–emitting diode lamps. (*c*) *Ex vitro*–acclimated shoots grown in commercial substrate in the greenhouse. *Scale bar*, 1 cm.

In *Vaccinium corymbosum*, using blue LED promoted accumulation of chlorophyll, while red LED reversed this effect (Hung *et al.* 2016). When using LED as a monochromatic source for grape plantlets, the isolated exposure to blue LED resulted in lower biomass, foliar area, and height, which was associated with reduced expression of the auxin-repressed protein gene, and increase in the carboxypeptidase serine protein (Li *et al.* 2017). Since blue and red spectra are required for chlorophyll synthesis and foliar growth, respectively, the combination of both spectra in a suitable proportion is important to obtain better plant growth and development results (Gupta and Jatothu 2013).

Axillary bud branching is another photomorphogenically controlled response preferentially accelerated by red LED light (Hung *et al.* 2016). Thus, although the accumulation of chlorophyll is important for the interception of radiation as mentioned, red LED lights, with wavelengths greater than those of the B LEDs, act in a complementary way during budding. Taken together, this evidence might help explain the greater shoot number and larger growth of these shoots formed under RB LED lamps (Figs. 3 and 5).

The acclimatization phase of *in vitro*–regenerated shoots is of great importance for a successful *in vitro* regeneration protocol. In this current study, the regulatory role of LEDs in modulating the metabolism of reactive oxygen species (ROS) by different LED lighting regimes, as reported by Gupta and Agarwal (2017), may have provided a basis for enhancing *in vitro* plant growth and increasing acclimatization survival.

Compared with reports by Fira *et al.* (2016), where the goji berry multiplication rate was 15.46 shoots per explant (regarding 1.5 to 2 cm long shoots), the present work maximized the multiplication to 58.17 to 77.56 shoots per explant. This represents at least a 32.4 to 76.5% multiplication rate increase, compared with the previous work. Another point to bear in mind is that Fira *et al.* (2016) achieved the multiplication rate from 4-node explants, while, here, in the current investigation, 3-node explants were inoculated.

The present study successfully developed an efficient *in vitro* multiplication protocol of economically important cultivars of goji berry, using a combination of optimal BAP concentration and RB LED illumination, to facilitate the large-scale commercialization of this species.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

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