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Research Article

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Enhancement of shoot organogenesis in *Polygonum tinctorium* by sucrose and gelling agents

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ABSTRACT

Indigo (*Polygonum tinctorium*), though it is a medicinal and dye crop, can also be grown in temperate areas, especially because it is commonly disseminated in Japan. To date, much research has been considered under investigation, especially for the regeneration of the indigo plant. Here in this study, we investigated the response of sucroses and gelling agents on the shoot organogenesis of the indigo plant. Micropropagation in terms of shoot regeneration and its growth was highly responded to sucrose and gelling agents. While culturing of internode explants on initial shoot regeneration media supplemented with sucrose and gelling agents of phytagar and gelrite significantly upgraded the regeneration efficiency as well as shoot growth. The regeneration capacity of the shoot was augmented with increased levels of sucrose up to 40 g L^{-1} and then started to decrease, whereas the increasing pattern continued even at the highest concentration (50 g L^{-1}). The highest shoot regeneration (6.0 ± 0.5) was achieved by the treatment of 40 g L^{-1} giving 7.5 times higher shoot regeneration compared to the control. The increasing pattern for shoot length was more pronounced than that of shoot regeneration. The shoot length ranged from 10.2 mm to 23.5 mm within the sucrose treatments. In this study, the highest shoot length (23.5± 0.21) was observed by the treatment of 50 g L^{-1} exhibiting 2.3 times higher shoot length compared to the control. Gelling agent gelrite performed better than phytagar for both regeneration and shoot length growth. The shoot regeneration among the phytagar treatments ranged from 3.8 to 6.0 shoots/explant whereas the shoot regeneration ranged from 6.6 to 7.2 among the gelrite treatments. The highest shoots/explant (7.2) and the longest shoot length (22.4 mm) were observed due to the treatment of gelrite 3. The lowest shoot regeneration and shoot length were denoted when phytagar 9 was applied. It is proposed from our study that sucrose and gelling agent especially gelrite 3 could be applied in shoot organogenesis and plant transformation of any plant species, especially for *P. tinctorium*.

KEYWORDS: Gelling agent, micropropagation, *Polygonum tinctorium*, Sucrose

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INTRODUCTION

Polygonum tinctorium, an annual herb has been used for a long time in the medicinal and dying industry (Cooksey, 2007; Hirota *et al.*, 2014) and has been utilized in traditional staining of fabric as a source of blue color in many countries, especially in Korea and Japan (Chung *et al.*, 2005; Hirota *et al.*, 2016). A compound, isolated from *P. tinctorium* named indigo has been extensively practiced in traditional Chinese medicine and also in the production of denim as a natural dye (Kukuła-Koch *et al.*, 2013). The highest amounts of indigo have been isolated from naturally occurring plant material through water extraction and alkaline precipitation for medicinal use (Campeol *et al.*, 2006). It has been reported that chronic myelocytic leukemia has efficiently been recovered using Indirubin, a key component of medicinal plants including *Polygonum tinctorium*, *Strobilanthes cusia*, and *Isatis tinctoria* (Hu *et al.*, 2015; Kumagai *et al.*, 2016).

It has also been used in traditional medicine for the improvement of atopic dermatitis (Han *et al.*, 2014), antioxidant (Tokuyama-Nakai *et al.*, 2018), anti-inflammatory (Micallef *et al.*, 2002), anticancer (Jang *et al.*, 2012), anti-HIV (Zhong *et al.*, 2005), antibacterial (Kataoka *et al.*, 2001), antidermatophytic (Honda *et al.*, 1980), and antifungal effect (Honda & Tabata, 1979).

Nowadays techniques of plant tissue culture have widely been used as an important tool for both basic and applied research and for mass production of economically important crops (Shasmita *et al.*, 2017; Ozdemir & Budak, 2018). Previously we successfully developed plant regeneration from stem internodes of *P. tinctorium* using various plant growth regulators, $AgNO_3$,

and putrescine through *in vitro* techniques (Thwe *et al.*, 2012; Kim *et al.*, 2016). In this study, we established a technique from internode explants by using different concentrations of sucrose and gelling agents for the regeneration of *P. tinctorium* plants.

MATERIALS AND METHODS

Seed Sterilization and Germination

Seeds of *Polygonum tinctorium* were attained from the own experimental farm of Chungnam National University, Daejeon, Korea. With the concentration of 70% (v/v) ethanol seeds were disinfected externally for 30 s and again kept in a 4.5% (v/v) aqueous solution of NaOCl with drops of Tween 20 for 15 min. Sterilized distilled water was used to wash the treated seeds under aseptic conditions and then kept on a solid basal medium for germination. To the basal Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) having salt and vitamins add 3% sucrose and then adjust the pH to 5.7-5.8. To this add 0.7% (w/v) plant agar and then sterilized by autoclaving at 121°C for 20 min. Approximately 10 sterilized seeds were kept in each Petri dish, and then incubated in a growth chamber maintained at the following temperature (25 ± 1°C) and illuminated at 35 µmol s^{-1} m^{-2} with 16-h photoperiod. One-week-old seedlings were shifted to a Magenta box (Magenta LLC, Chicago, IL, USA) having the same MS solid medium and continued the growth of the plants for 3 weeks.

Improvement of *In Vitro* Regeneration

Stem segments *in vitro* containing one internode (approximately 1.0 cm in length) of *P. tinctorium* were cut aseptically. The explants were allowed to grow on a medium comprising 50 mL of the respective medium. For shoot regeneration, the MS medium was supplemented with 2 mg L^{-1} 6-benzyl amino purine (BAP). The media were optimized by adding sucrose at different concentrations (0, 10, 20, 30, 40, and 50 g L^{-1}) for enhancement of shoot regeneration. Here in this study concentrations of the gelling agents (6, 7, 8, and 9 g L^{-1} of phytagar and 2, 3, 4, and 5 g L^{-1} of gelrite) were investigated for efficient shoot formation. All the chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The explants were allowed to grow in a growth chamber maintain at 25 ± 1°C, with a 16-h photoperiod, and illuminated at 35 µmol s^{-1} m^{-2} for 5 weeks.

Rooting of Regenerated Shoots

For root induction, the regenerated shoots (~2.0 cm long) were moved to a rooting medium. The rooting MS medium was gelled with 8 g L^{-1} of plant agar along with 1 mg L^{-1} indole-3-butyric acid (IBA) and autoclaved the medium. After autoclaving, the medium was allowed to cool and then poured into a 50 mL culture vessel and allow to solidify. The next day, four shoots were placed in each culture vessel. Then the culture vessel was moved into a growth chamber, maintained at 25 ± 1° with a 16-h photoperiod and illuminated at 35 µmol s^{-1} m^{-2} for 4 weeks. Then the rooted plants were cleaned with sterile water to eliminate plant agar and then moved into pots. The pots were filled with autoclaved vermiculite, and the plants were moved into the pot and then covered with polyethylene bags for one week to maintain high humidity. The plants were incubated in a growth chamber with a 16-h photoperiod and a night/day temperature of 18/20°C for two weeks. After one week the polyethylene cover was removed. It is assumed that within two weeks these plants adjusted to be replaced at the greenhouse for further use.

Statistical Analysis

The statistical analyses were done by using SPSS 26.0 (IBM Corp., NY, USA). The data obtained were presented as means ± standard deviation of 50 stem internodes tested.

RESULTS AND DISCUSSION

Effect of Various Concentrations of Sucrose and Gelling Agents on Shoot Regeneration and Shoot Length

A positive response was denoted due to the addition of sucrose for regeneration and elongation of the shoot. It is denoted from this study that the regeneration capacity of the shoot increased as concentrations of sucrose increased and it continued up to 40 g L^{-1} and then started to decrease, whereas the increasing pattern continued even at the highest concentration (50 g L^{-1}) used in this study (Figure 1). The range of shoot regeneration was 0.8 to 6.0 within the treatment. The highest shoot regeneration (6.0± 0.5) was achieved by the treatment of 40 g L^{-1} giving 7.5 times higher shoot regeneration compared to the control. In the control, the shoot regeneration was only 0.8. The shoot increment rate was 4.25 times higher just after using the first sucrose treatment 10 g L^{-1} next to the control. A similar pattern was followed for the shoot length in response to concentrations of sucrose. The increasing pattern for shoot length was more pronounced than that of shoot regeneration. Here the increasing pattern continued even at the highest level of sucrose. The shoot length ranged from 10.2 mm to 23.5 mm within the sucrose treatments. In this study, the highest shoot length (23.5 ± 0.21) was observed by the treatment of 50 g L^{-1} exhibiting 2.3 times higher shoot length compared to the control. The lowest shoot length was 10.2 which were found in the control treatment.

Shoot regeneration and shoot length in explants are enhanced while culturing in media with the addition of gelling agents of phytagar and gelrite in different concentrations. There is no distinct pattern observed among the treatments for shoot regeneration and shoot length. The range of shoot regeneration lies from 0.8 to 7.2 in response to gelling agent treatment. Gelling agent gelrite performed better than phytagar in both regeneration and shoot length growth. The shoot regeneration among the phytagar treatments ranged from 3.8 to 6.0 shoots/explant whereas the shoot regeneration ranged from 6.6 to 7.2 among the gelrite treatments (Figure 2). The highest shoots/explant (7.2) was observed due to the treatment of gelrite 3 giving 9.0 times higher shoot regeneration compared to the control. Among the gelling agent's treatments, phytagar 9 produced the lowest shoot regeneration (3.8 shoots/explant). The shoot length ranged from 10.2 to 22.4 mm among the treatments. The shoot length ranged from 19.0 to 22.4 mm

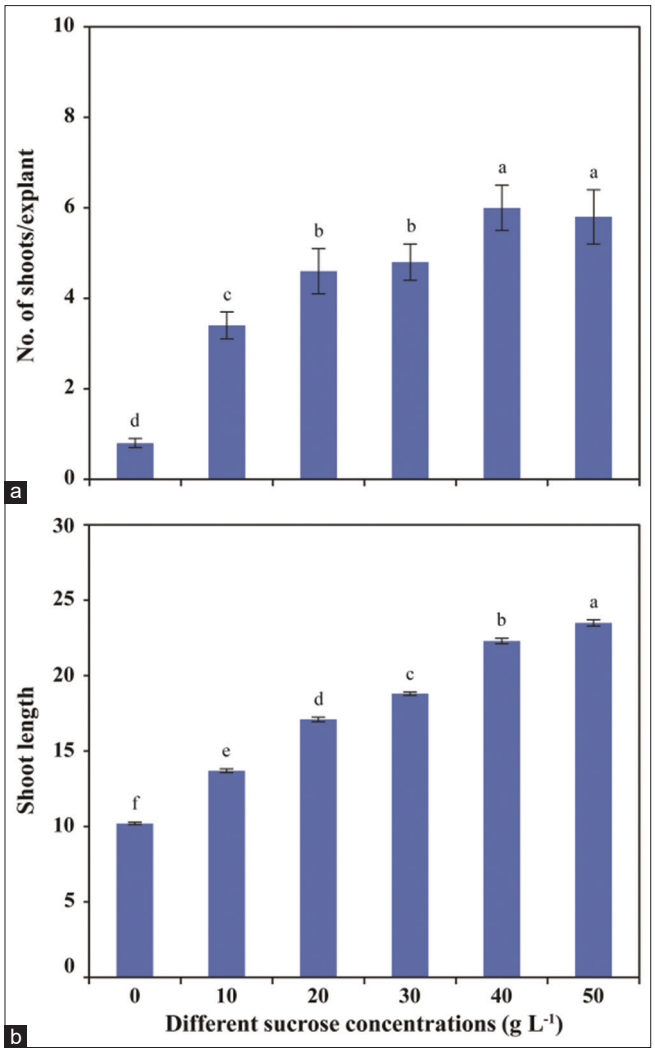

Figure 1: Effect of various concentrations of sucrose on shoot regeneration from stem internode explant cultures of *Polygonum tinctorium* after 5 weeks of culture on MS medium supplemented with 2 mg L^{-1} BAP (shoot regeneration medium). Values represent the mean ± standard deviation of 50 shoots

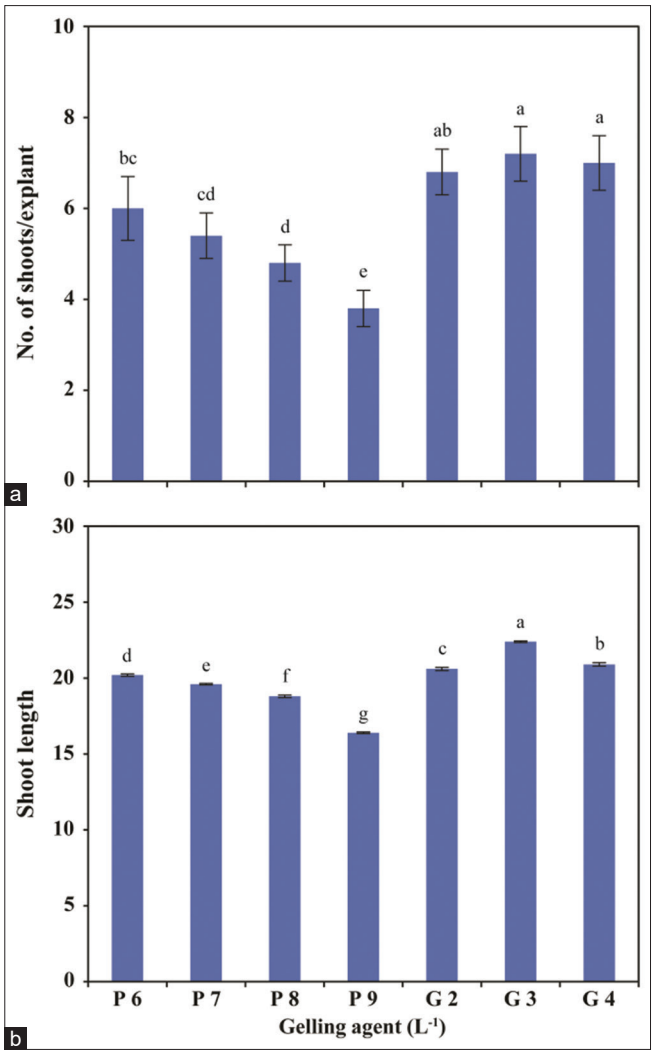

Figure 2: Effect of various concentrations of gelling agents on shoot regeneration from stem internode explant cultures of *Polygonum tinctorium* after 5 weeks of culture on MS medium supplemented with 2 mg L^{-1} BAP (shoot regeneration medium). Values represent the mean ± standard deviation of 50 shoots. P-phytagar; G-gelrite

among the gelrite treatments, and it was 16.4 to 20.2 mm in shoot length among the phytagar treatments. The highest shoot length was found when gelrite 3 was treated exhibiting 2.2 times higher shoot length compared to the control treatment. As for shoot regeneration the gelling agent treatments, phytagar 9 produced the lowest shoot length (16.4 mm).

Sucrose usually acted as the main transport sugar in the phloem sap of most plants. It was reported that the morphogenetic potential of plant tissues is greatly influenced by different sources of carbon in the micropropagation systems (Yaseen *et al.*, 2013). It was proclaimed that a maximum number of shoot organogenesis in *Aerva lanata* (23.6 ± 0.16) was observed after 21 days of culture incubation on a medium containing 1.0 mg L^{-1} thidiazuron (Varutharaju *et al.*, 2014). Plant regeneration through organogenesis was noted on the MS salts supplemented with 2 mg L^{-1} 2,4-dichlorophenoxyacetic acid, 0.01 mg L^{-1} BAP, and 6% and 3% sucrose for the formation of shoot and root, respectively. By illuminating at 45 µmol s^{-1} m^{-2} the highest regeneration capacity (50 plantlets per callus) was found (Ncanana *et al.*, 2005). For the regeneration of sugarbeet plants from hypocotyl explants (at 21 day-old-seedlings), a simple and reproducible protocol has already been developed. Explants were cultured on MS medium using different sources of carbons like 0.5% (w/v) fructose, 0.5% (w/v) glucose, and 0.5% (w/v) sucrose to induce the formation of organogenic calli. It was reported that shoot regeneration was initiated in callus cultures of more than 1600 genotypes (Jacq *et al.*, 1992). It was observed that 2% sucrose supplemented in MS medium was the best for embryogenesis whereas 4% sucrose was the best for shoot bud differentiation in *Albizia richardiana* (Tomar *et al.*, 1988). Initiation of the shoot was observed after 4 weeks on calli shifted to light on a medium consisting of 10% coconut milk, 0.5 mg L^{-1} 6-benzyl adenine, and 20 g L^{-1} sucrose in *Eucalyptus camaldulensis* Dehnh (Muralidharan & Mascarenhas, 1987).

Gelrite was found to be the most effective gelling agent in promoting the shoot in different chrysanthemum cultivars (Lim *et al.*, 2012). Phytagel provided the highest shoot weight (overall mean = 2.4 g), while the lowest shoot weight was 1.7, 2.2, and 2.2 g, in the treatment of agar, agar gel, and plant agar, respectively of Dwarf Cavendish bananas (Kaçar *et al.*, 2010). The phytagel and agargel media boosted the highest mean shoot length. In a study, banana shoots were cultured with gelling agents such as gelrite, agar, and gellan gum for 13 weeks. It was observed that each of the gelling agents showed enhanced plant growth. But shoot growth and multiplication rate were higher on the medium gelled with 0.9 g L^{-1} gelrite as compared with those on the medium solidified with 2-6 g L^{-1} gellan gum or 4-8 g L^{-1} agar (Buah *et al.*, 1999). The highest growth of shoots and roots of orchid (*Dendrobium sonia*) was observed by using isubgol as an alternative gelling agent on agar at 30 g L^{-1}. The cost of 30g L^{-1} isubgol (500g costs US$ 10 only) was much cheaper than conventionally used agar. An alternative gelling agent might be considered a new possibility of using a low-cost agent which will lessen the production costs considerably in plant tissue culture techniques (Ullah *et al.*, 2015). It was reported from the study of an *in vitro* seed germination, shoot differentiation, and rooting of *Albizzia lebbeck* on media solidified either with 2.6% isubgol+0.4% agar, were similar to or ameliorate than the controls. Xanthagar [0.6 % xanthan gum+0.4 % agar] gelling mix is suggested as a possible alternative source to agar because of its suitability comparable to agar and its distinct cost advantage (Jain-Raina & Babbar, 2011).

CONCLUSIONS

For micropropagation in plants, the development of an efficient protocol is the most important factor for being used for the induction of genetic transformation and plant growth improvement. Nowadays shoot organogenesis is the most commonly used procedure for *in vitro* plant regeneration. In this study, the developed protocol showed the highest number of regenerations was achieved in *P. tinctorium*. The carbon source from sucrose and gelling agents of phytagar and gelrite stimulated the efficiency of shoot organogenesis and shoot elongation in this species. Our findings would be worked well for the genetic improvement of many medicinal plants including *P. tinctorium*.

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