In Vitro Shoot Regeneration from Stem Internodes of *Polygonum tinctorium*

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Abstract: An efficient and simple protocol for shoot organogenesis and plant regeneration from the stem internodes of *Polygonum tinctorium* was developed. The stem internodes were cultured on media containing different concentrations of cytokinins. Thereafter, the optimum concentration of cytokinin was selected and supplemented with various concentrations of auxins, to determine the potential regeneration capacity and shoot growth from excised stem internodes. Of the cytokinins, 6-benzylaminopurine exhibited the highest efficiency for shoot initiation, followed by zeatin, kinetin, and 1-phenyl-3-(1, 2, 3-thiadiazol-5-yl)-urea. In this study, 2.0 mg/L of 6-benzylaminopurine was highly effective for shoot initiation, in terms of regeneration efficiency, shoot number, and shoot length. Among the cytokinin/auxin combinations, 2.0 mg/L of 6-benzylaminopurine with 0.5 mg/L indole-3-butyric acid provided the optimal conditions for yielding the highest number of shoots (5.6) per explant, greatest shoot length (19.8 mm), and the highest regeneration rate (98%). The protocol developed here is potentially useful for studying the *in vitro* organogenesis of *P. tinctorium.*
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1. Introduction

Polygonum tinctorium, which is commonly known as subtropical indigo, is an herbaceous annual plant belonging to the family Polygonaceae (Angelini et al., 2004). The indigo plant has been regularly used as an important source of dye, due to the beautiful blue color that is produces (Jang et al., 2012; Gilbert et al., 2004). The indigo plant has various pharmacological and medicinal properties, exhibiting antianaphylactic, antibacterial, antiviral, anti-inflammatory, cytotoxic, and apoptotic actions (Kim et al., 1998; Ishihara et al., 2000; Kataoka et al., 2001; Zhong et al., 2005). Moreover, anti-tumor and anti-oxidative activities have been identified in ethyl acetate extracts of this plant. According to observations, the anti-tumor and anti-leukemia activities are attributable to tryptanthrin, whereas the anti-oxidative activity has been attributed to gallic acid and caffeic acid, which are also present in ethyl extracts of the indigo plant (Kimoto et al., 2001; Koya-Miyata et al., 2001; Honda et al., 1980).

It is possible to propagate indigo plants from seeds in the natural environment. However, this method requires time, with scarification (i.e., breaching the seed coat by mechanical, thermal, or microbial techniques) sometimes being necessary because of the plant's hard seed coat. Therefore, *in vitro* propagation and regeneration has been developed for the effective use of *in vitro* grown plants that are used for many purposes in molecular biology. Plant tissue culture is the *in vitro* culture of plant cells, tissues, and organs, and this process may regenerate the whole plant in the culture medium. This technique serves an important tool for both basic and applied studies, as well as in the commercial production of economically important crops (Chandra et al., 2010; Debnath et al., 2006; Thorpe, 2007). The technique also provides an effective means for studying the rapid propagation of plants, because of advantages such as a large propagation coefficient, short reproductive cycle, and year-round production and supply (Nge et al., 2006). An efficient technique for the regeneration of plant parts via tissue culture is therefore critical for multiple micropagation (i.e., rapidly multiplying stock) and genetic transformation. In this study, we developed an efficient method for the regeneration of *P. tinctorium* plants by using different quantities and combinations of plant hormones.

2. Experimental Procedures

Seed Sterilization and Germination

Seeds of *P. tinctorium* were procured from the experimental farm of Chungnam National University (Daejeon, Korea). The seeds were disinfected with 70% (v/v) ethanol for 30 s and 4.5% (v/v) sodium hypochlorite aqueous solution, together with a few drops of Tween 20 for 15 min. Subsequently, the seeds were thoroughly rinsed with sterilized distilled water under aseptic conditions, and then cultured on a solid basal medium for germination. The basal medium consisted of Murashige and Skoog (MS, Murashige and Skoog, 1962) salt and vitamins

supplemented with 3% sucrose, and solidified with 0.7% (w/v) plant agar. The medium was adjusted to pH 5.8 before adding the plant agar, and was then sterilized by autoclaving at 121°C for 20 min. Ten seeds were placed on each Petri dish, and these were placed in a growth chamber at 25 ± 1°C under standard cool white fluorescent tubes with a flux rate of 35 µmol s^{-1} m^{-2} and 16-h photoperiod. The seeds germinated within 1 week of culture, and the seedlings were then transferred to a Magenta box containing the same MS solid medium, and cultured for 3 weeks for the establishment of plant material.

***In Vitro* Plant Regeneration**

Stem segments of *P. tinctorium*, approximately 2.0 cm in length, and each including one internode, were aseptically cut from plants grown *in vitro*. The explants were cultured on 50 mL of the respective medium in a Magenta box. For shoot regeneration from stem internodes, the MS medium was supplemented with 0, 0.1, 0.5, 1, 2, or 4 mg/L BAP (6-benzylaminopurine), kinetin (N^6-furfuryladenine), TDZ (1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea; thidiazuron), and zeatin. To improve shoot regeneration, the medium was optimized by testing the effect of different concentrations of 0, 0.1, 0.5, and 1.0 mg/L IAA (indole-3-acetic acid), IBA (indole-3-butyric acid), and NAA (α-naphthalene acetic acid) in combination with 2 mg/L of BAP on shoot formation and growth. Plant hormones were purchased from Sigma Chemical Corporation (St. Louis, MO, USA). Cultures were maintained at 25 ± 1°C in a growth chamber with a 16-h photoperiod under standard cool white fluorescent tubes (35 µmol s^{-1} m^{-2}) for 5 weeks.

Rooting of Regenerated Shoots

Regenerated shoots (~2.0 cm long) were placed in MS medium for root induction. The medium was solidified with 8 g/L of plant agar and dispensed at 50 mL per culture vessel, with 4 shoots being cultured in each culture vessel. Regenerated shoots were incubated at 25 ± 1°C in a growth chamber with a 16-h photoperiod under standard cool white fluorescent tubes (35 µmol s^{-1} m^{-2}) for 4 weeks. After 4 weeks of culture, the rooted plants were washed with sterile water to remove plant agar, transferred to pots containing autoclaved vermiculite, and covered with polyethylene bags for 1 week to maintain high humidity. The plants were then transferred to soil, and maintained in a growth chamber with a 16-h photoperiod, and a night/day temperature of 18–20°C for 2 weeks. These hardened plants were then transferred to the greenhouse for further use.

Statistical Analysis

The experimental data derived from the 50 tested stem internodes were expressed as the mean ± standard deviation.

3. Results and Discussion

Effect of Cytokinins on Shoot Growth

The rapid development of *in vitro* shoot regeneration from the stem internodes of *P. tinctorium* was investigated. The efficiency of shoot regeneration, shoot number, and shoot length were determined by using MS basal medium supplemented with different concentrations of cytokinins. Shoot development was successful under all cytokinin regimes. Increased shoot regeneration was observed with increasing concentrations of BAP, kinetin, and zeatin from 0.1 to 2.0 mg/L for all measured parameters. However, shoot regeneration decreased significantly when these cytokinin concentrations were raised to 4 mg/L (Table 1). The trend for TDZ was slightly different compared to the other cytokinin groups. This hormone produced a gradual increase in shoot organogenesis up to 1.0 mg/L of TDZ concentration, and decreased beyond this. At a concentration of 2.0 mg/L, BAP produced the highest frequency of shoot formation (96%), the maximum number of adventitious shoots (5.2) per explant, and the longest shoot lengths (18.8 mm), compared to all other concentrations of BAP and compared to the other cytokinins used in this study.

The plant hormone, BAP, has also been found to be the most effective hormone for shoot organogenesis in other plants, such as *Nicotiana tobacum* (Marcotrigiano, 1986) and *Cucumis melo* (Marta and Andrés, 2009). Eckardt (2003) reported that cytokinin deficiency causes a major decline in shoot development, consequently leading to dwarfism, late flowering plants, enhanced root growth, and changes in reproductive development. Furthermore, cytokinins are required in plant tissues to maintain both cell division and leaf primordium formation. Therefore, cytokinins are considered important hormones for plant growth.

Shoot Promotion by Auxins in Combination with Cytokinin

Previously cultured *P. tinctorium* stem internodes were grown for 5 weeks on MS basal medium containing 2.0 mg/L BAP supplemented with various concentrations (0,0.1,0.5, and 1.0 mg/L) of different auxins (IAA, IBA, and NAA) to evaluate shoot regeneration and multiplication of stem internodes. Among the treatments, 0.5 mg/L IAA and 0.5 mg/L IBA in combination with cytokinin (BAP 2.0 mg/L) produced more shoots from the explant compared to the control (cytokinin alone). At all concentrations of auxins, NAA exhibited a negative response with the cytokinin combination (Table 2). As NAA concentration increased from 0.1 to 1.0 mg/L, it caused a decreasing trend in shoot growth. Moreover, the lowest regeneration efficiency, shoot number (3.6 shoots per explant), and shortest shoot length (13.2 mm) were observed at 1.0 mg/L NAA compared to all other auxin treatments.

Table 1. Effect of different concentrations of cytokinins on the shoot regeneration of stem internode explant cultures of *Polygonum tinctorium* after being grown for 5 weeks on MS medium

Cytokinins (mg/L)		Regeneration (%)	Shoot no. per explant	Shoot length (mm)
BAP	0.1	72	2.4 ± 0.1	13.0 ± 0.07
	0.5	84	4.2 ± 0.3	14.6 ± 0.09
	1	94	4.8 ± 0.3	18.0 ± 0.10
	2	96	5.2 ± 0.2	18.8 ± 0.08
	4	81	2.4 ± 0.1	16.0 ± 0.07
Kinetin	0.1	74	2.0 ± 0.2	12.4 ± 0.05
	0.5	79	3.6 ± 0.1	13.4 ± 0.05
	1	84	4.0 ± 0.2	17.0 ± 0.07
	2	89	4.2 ± 0.3	16.0 ± 0.12
	4	85	2.0 ± 0.2	12.6 ± 0.09
TDZ	0.1	68	1.8 ± 0.1	11.8 ± 0.08
	0.5	75	2.6 ± 0.1	13.4 ± 0.15
	1	89	2.8 ± 0.3	17.4 ± 0.09
	2	75	2.4 ± 0.1	17.6 ± 0.05
	4	53	1.6 ± 0.1	14.2 ± 0.08
Zeatin	0.1	76	1.4 ± 0.1	12.4 ± 0.05
	0.5	85	2.4 ± 0.1	13.2 ± 0.08
	1	88	3.4 ± 0.3	16.4 ± 0.09
	2	92	4.6 ± 0.1	18.4 ± 0.05
	4	79	3.6 ± 0.1	16.2 ± 0.08

Table 2. Effect of different concentrations of auxins on the shoot regeneration of stem internode explant cultures of *Polygonum tinctorium* after being grown for 5 weeks on shoot regeneration medium (MS medium with 2 mg/L BAP)

Auxins	(mg/L)	Regeneration (%)	Shoot no. per explant	Shoot length (mm)
Control	0.0	96	5.2 ± 0.2	18.8 ± 0.08
IAA	0.1	95	5.0 ± 0.4	17.8 ± 0.08
	0.5	96	5.4 ± 0.3	18.2 ± 0.08
	1	97	5.0 ± 0.1	17.4 ± 0.05
IBA	0.1	97	4.8 ± 0.2	18.4 ± 0.11
	0.5	98	5.6 ± 0.1	19.8 ± 0.10
	1	96	5.2 ± 0.1	17.0 ± 0.12
NAA	0.1	95	5.2 ± 0.2	18.0 ± 0.12
	0.5	93	5.0 ± 0.1	15.6 ± 0.09
	1	86	3.6 ± 0.1	13.2 ± 0.08

Therefore, NAA did not enhance shoot growth in this study. Overall, the highest number of shoots (5.6 shoots per explant), longest shoot length (19.8 mm), and the best regeneration efficiency (98%) were obtained from 0.5 mg/L IBA in combination with 2.0 mg/L BAP.

In this study, BAP and IBA played an important role in the shoot regeneration of *P. tinctorium*. It is well known that cytokinins stimulate plant cell division and contribute to the release of lateral bud dormancy, induction of adventitious bud formation, growth of lateral buds, and cell cycle control. In comparison, auxins exert a strong influence on the initiation of cell division, meristem organization giving rise to un-organized tissue (callus) or defined organs (shoots), cell expansion, cell wall acidification, apical dominance, promotion of vascular differentiation, and root formation (Gaspar et al.,

1996). Therefore, manipulation of the exogenous cytokinin:auxin balance might influence the pattern of plant development, thus facilitating the development of an organogenic program (Gaspar et al., 2003).

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