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BRIEF COMMUNICATION

Efficient regeneration of tetraploid *Isatis indigotica* plants via adventitious organogenesis from hypocotyl explants

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Abstract

An efficient *in vitro* plant regeneration system via hypocotyl segments of tetraploid *Isatis indigotica* Fort. was established. Murashige and Skoog's (MS) and Gamborg's (GB_5) media were found to be superior to White medium for promoting shoot regeneration. The highest shoot regeneration (92 %) was achieved from hypocotyls cultured on MS medium containing 8.9 μM benzyladenine (BA) and 2.7 μM naphthaleneacetic acid (NAA), with an average of 4.2 shoots developed per explant. Plant regeneration was also improved when the explants were cultured in MS basal medium containing 3 % (m/v) sucrose and grown under a 12-h photoperiod. The developed shoots were well rooted in a half-strength MS medium supplemented with 0.5 μM indole-3-butyric acid (IBA) and were morphologically normal after transfer to soil.

Additional key words: growth regulators, medicinal plant, plant regeneration, tissue culture.

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Isatis indigotica Fort. is a medicinal plant belonging to family *Cruciferae*. It is self-incompatible with small yellow flowers and is the major ingredient of traditional Chinese medicines. Leaves of the autotetraploid *I. indigotica* (2n=28) contain much more of the effective ingredients such as indigo, indurubin and total amino acids than those of its diploid parent (2n=14) (Qiao *et al*. 1995, Wang *et al*. 2000). Due to its active substances, tetraploid *I. indigotica* is in ever-growing demand and now in great shortage. Propagation via tissue culture which has been successful in some other species (Gamborg 2002) is one of the efficient ways to solve the shortage problem.

Although diploid *I. indigotica* regeneration from mesophyll protoplasts (Hu *et al*. 1999), calluses (Chen *et al*. 2000) and hypocotyls (Peng and Wang 1994) has been reported, there is no previous report on plant regeneration from tetraploid *I. indigotica* by tissue culture. As plants regenerated via calluses may be genetically less stable than those obtained through direct organogenesis, direct adventitious shoot organogenesis is preferred (Singh *et al*. 2002). Previous studies showed that many factors influenced culture and regeneration of plants *in vitro*, of which the choice of medium types and the use of plant growth regulators were among the most influential (Bischoff and Mahn 2000, Tang *et al*. 2000). In this paper, we report the successful direct plant regeneration from hypocotyl explants of tetraploid *I. indigotica*. Factors influencing plant regeneration, including plant growth regulators, formulation of salts and vitamins, and other factors including sucrose concentration and illumination were investigated.

Seeds of tetraploid *I. indigotica* Fort. were kindly provided by Prof. C.Z. Qiao from School of Pharmacy at

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Abbreviations: BA - 6-benzyladenine; GB_5 - medium of Gamborg; IBA - indole-3-butyric acid; NAA - α-naphthaleneacetic acid; MS - mediun of Murashige and Skoog (1962); White - medium of White (1943).
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the Second Military Medical University, Shanghai, China. Seeds were surface sterilized by immersing in 70 % (v/v) ethanol for 1 min, followed by 15 min in 0.1 % (v / v) $HgCl_2$. After several rinses with sterile distilled water, the seeds were placed onto 20 cm^3 of hormone-free MS medium (Murashige and Skoog 1962) containing 3 % sucrose and solidified with 2.6 g dm^{-3} *Phytagel* (*Sigma*, St. Louis, USA) in 9-cm Petri dishes (10 seeds per plate) for germination.

Hypocotyls were excised from one-week-old *in vitro* plantlets, and segments (about 0.5 cm long) were used as explants in this study. Four experiments were carried out to test the effect of plant growth regulators, culture medium type, sucrose and irradiance on shoot regeneration from hypocotyl segments. All the culture media were solidified with 2.6 g dm^{-3} *Phytagel* and adjusted to pH 5.8 prior to autoclaving for 20 min at 121 °C. The cultures were incubated at 25 ± 2 °C. Three weeks later, the cultures were transferred to the same fresh medium for further two weeks of growth. Developed shoots (approximately 3 cm in height) were excised from shoot bases and transferred to rooting medium (half-strength MS medium supplemented with 0.5 μM IBA, 3 % sucrose and solidified with 2.6 g dm^{-3} *Phytagel*, pH 5.8) under 12-h photoperiod (white fluorescent tubes: irradiance of 40 μmol m^{-2} s^{-1}) for root development. Two weeks later, plantlets with vigorously growing roots were transplanted and acclimated in soil in greenhouse.

To test the effect of plant growth regulators on shoot regeneration, cotyledon segments were cultured on MS basal medium containing different concentrations and combinations of BA and NAA (Table 1). All the media contained 3 % sucrose and the explants were cultured under the conditions as for seed germination. To test the effect of culture medium type on shoot regeneration, three of the most commonly used plant culture media, MS, GB_5 (Gamborg 1970) and White (White 1943) were selected. All the media contained 2.0 mg dm^{-3} (8.9 μM) BA, 0.5 mg dm^{-3} (2.7 μM) NAA and 3 % sucrose, and the explants were incubated as previously described. To test the effect of sucrose on shoot regeneration, sucrose at concentrations ranging from 1 to 5 % (m/v) was added in MS basal medium containing 8.9 μM BA and 2.7 μM NAA. To test the effect of irradiance on shoot regeneration, two groups of cotyledon explants were cultured under 12-h photoperiod (40 μmol m^{-2} s^{-1}) or in continuous darkness. All the explants were cultured on MS basal medium containing 8.9 μM BA, 2.7 μM NAA and 3 % sucrose.

Forty explants were used for each treatment, and each treatment was repeated three times. The number of adventitious shoots per explant was recorded. Plant regeneration frequency was calculated as the percentage of total numbers of explants producing shoots in total numbers of explants inoculated. The experimental design was a completely randomized design (CRD) and the data were analyzed using *ANOVA* for CRD. Fisher's least significant difference (LSD) was used to compare the means and standard deviations (SD) of the means were calculated.

Table 1. Influence of plant growth regulators on plant regeneration from hypocotyl explants of tetraploid *I. indigotica.* Means ± SD based on three replicates, 40 explants per replicate (*i.e.* 120 explants were tested for shoot regeneration). For shoot regeneration differences among the different treatment and the control were significant at $P < 0.05$; r - roots developed from some explants; c - some explants developed calluses.

BA [μM]	NAA [μM]	Regeneration frequency [%]	Root	Callus	Total plants regenerated
0.0	0.0	0	-	-	0
0.9	2.7	26.8 ± 1.6	r	-	32
0.9	5.4	44.5 ± 5.8	r	-	54
0.9	10.7	78.6 ± 6.3	r	-	94
0.9	26.9	0	r	c	0
4.4	2.7	87.0 ± 2.2	-	-	104
4.4	5.4	85.3 ± 5.8	-	-	102
4.4	10.7	77.7 ± 3.4	-	-	93
4.4	26.9	0	-	c	0
8.9	2.7	92.0 ± 4.1	-	-	110
8.9	5.4	86.9 ± 2.7	-	-	104
8.9	10.7	79.2 ± 3.3	-	-	95
8.9	26.9	0	-	c	0
22.2	2.7	38.6 ± 3.2	-	-	46
22.2	5.4	43.5 ± 3.6	-	-	52
22.2	10.7	28.7 ± 3.2	-	-	34
22.2	26.9	0	-	c	0

Plant growth regulators and their combinations significantly influenced shoot regeneration from hypocotyl segments of tetraploid *I. indigotica* (Table 1). The 8.9 μM BA was most suitable for shoot regeneration. When the concentration of BA in the medium was reduced to 0.9 μM, some explants developed roots, instead of shoots. When the concentration of BA in the medium was raised to 22.2 μM, shoot regeneration from hypocotyls declined significantly, no matter what concentration of NAA was present in the medium. A combination of 8.9 μM BA and 2.7 μM NAA was found to be best for promoting shoot regeneration with the regeneration frequency of 92 % (Table 1). Multiple shoots (0.2 - 0.5 cm in length) developed 1 - 2 weeks later with an average of 4.2 shoots per explant. Adventitious shoot regeneration was also noticed in the culture of diploid *I. indigotica* although the number of shoots per explant was not given (Peng and Wang 1994). The percentage of hypocotyl explants that formed shoots represents the response capacity of the explant to the medium and the number of shoots per explant represents the capacity of the explants to produce shoots, which has been found to be influenced by many factors such as

explant type (Pablo *et al.* 2002), medium composition and hormone combination in the medium (Bischoff and Mahn 2000, Svetleva *et al.* 2001, Yokoya and Handro 2002) and culture condition (Chen and Chang 2002).

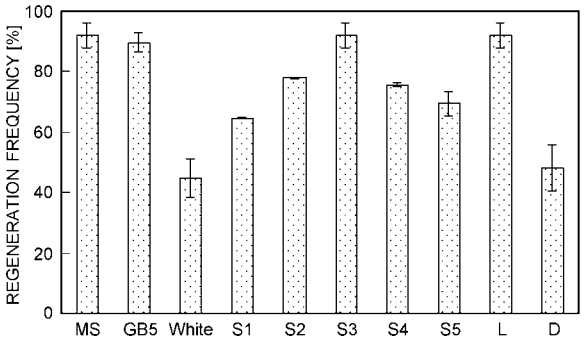

Fig. 1. Influence of medium type, sucrose concentration and irradiation on plant regeneration from hypocotyl explants of tetraploid *I. indigotica*. MS - Murashige and Skoog medium. GB5 - Gamborg's B_5 medium. White - White′s medium. The explants were cultured on media containing 8.9 μM BA, 2.7 μM NAA and 3 % sucrose under 12-h photoperiod. S1 - S5: the explants were cultured on MS basal medium containing 8.9 μM BA, 2.7 μM NAA and sucrose at concentration from 1 to 5 % (m/v) under 12-h photoperiod. L,D: the explants were cultured on MS basal medium containing 8.9 μM BA, 2.7 μM NAA, and 3 % sucrose under 12-h photoperiod (L) or under darkness (D). *Vertical bars* represent SD of the means, $n = 40$.

Different culture media which contained different macro salts and total nitrogen content were found to have substantial influence on the growth of explants (Vanegas *et al.* 2002, Zheng *et al.* 2002, Yokoya and Handro 2002). Previous study revealed that the amount and type of nitrogen source had significant effect on tissue cultures (Chen and Chang 2002). The macro salt and total nitrogen content were different in the three basal media (MS, GB_5 and White) used in the study. The total nitrogen content in White (3.3 mg dm^{-3}) was much lower than that in MS (60.0 mg dm^{-3}) and GB_5 (26.7 mg dm^{-3}). The frequencies of hypocotyl explants that produced shoots on MS (92 %) and GB_5 (89.6 %) media were found to be significantly higher ($P < 0.05$) than that on White medium (44.6 %) and the highest shoot regeneration frequency was achieved when MS medium was used (Fig. 1). This result indicates that higher total nitrogen content is beneficial for shoot regeneration from hypocotyl explants of tetraploid *I. indigotica*. This result is also supported by a previous report in which MS was found to be the best medium to support callus and shoot induction from hypocotyl explants of diploid *I. indigotica* (Peng and Wang 1994).

Most media used in plant tissue culture contain sucrose as carbon and energy source and the concentration of sucrose was found to be influential to plant regeneration (Chen and Chang 2002). The present study showed that although shoot regeneration could be achieved from hypocotyl explants when cultured on the medium containing various concentrations of sucrose ranging from 1 to 5 %, 3 % was the sucrose concentration that supported the highest shoot regeneration from hypocotyl explants (Fig. 1). We also observed that plant regeneration frequency was significantly enhanced when the explants were cultured under 12-h photoperiod, reaching over 90 %, compared with less than 50 % under continuous darkness ($P < 0.05$). Plantlets grown under light were sturdy with well-developed green leaves. When transferred to half-strength MS medium supplemented with 0.1 mg dm^{-3} IBA, approximately 96 % of shoots (3 cm high) developed roots with an average rooting efficiency of 3 roots per shoot. When transferred to soil in pots in the glasshouse, 162 out of 200 plantlets derived from explants cultured on MS basal medium containing 8.9 μM BA, 2.7 μM NAA and 3 % of sucrose were successfully acclimatized and eventually established in soil. The percentage of survival of the plantlets as recorded after 4 weeks of transfer to soil was over 80 %. The regenerants were morphologically normal.

In the present study, plant regeneration could be achieved from explants after 1 - 2 months of culture. The establishment of efficient *in vitro* plant regeneration system from hypocotyl explants of tetraploid *I. indigotica* provides an effective way to meet ever-growing demands for tetraploid *I. indigotica*. In addition, *in vitro* shoot regenerants obtained from the current study could serve as an effective source for protoplasts for genetic improvement.

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