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Full Length Article

Effects of Agar and Isubgol on Adventitous Shoot Regeneration of Woad (*Isatis tinctoria*)

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ABSTRACT

A practical, effective and economic shoot regeneration system was developed using a cheap alternative gelling substance ısubgol for use in *in vitro* regeneration of woad (*Isatis tinctoria* L.) plant. The leaf and hypocotyl explants belonging to woad were cultured on Murashige and Skoog (MS) medium solidified with 6 g/L agar and 15 g/L isubgol containing different concentrations of BAP-NAA. Maximum shoot regeneration of 12.65 and 17.80 shoot per leaf explant on agar and isubgol gelled medium was recorded on MS medium containing 1.00 mg/L BAP+0.25 mg/L NAA and 0.50 mg/L BAP, respectively. Maximum shoot regeneration of 19.87 and 20.55 shoots per hypocotyl explant on agar and isubgol gelled medium was recorded on MS medium containing 0.50 mg/L BAP+0.25 mg/L NAA and 1.00 mg/L BAP, respectively. Isubgol revealed better shoot regeneration used as gelling agent as compared to agar using same nutrient medium. Moreover more shoots were recorded on MS medium containing BAP only compared to medium containing both BAP-NAA. Compared with agar gel, isubgol is cheaper gelling agent, which can be used to reduce the cost of tissue culture. © Friends Science Publishers

Key Words: *Isatis tinctoria;* Adventitious shoot regeneration; Isubgol; Economy

INTRODUCTION

Isatis tinctoria L. (woad, family Brassicaceae) is a perennial dye and medicinal herb and is native to the steppe and desert zones of the Central Asia to Eastern Siberia, Western Asia, South West Asia (Shu, 2001) and Europe (Tan, 2002). It was widely cultivated in Europe from the 12th to the 17th century as a source of indigo (Hurry, 1930; Clark *et al.*, 1993). The plant was largely grown in England (Somerset, Lincolnshire), France (Normandy, Somme, Languedoc), Germany (Ju¨ lich, Thuringia) and Italy (Piedmont, Tuscany) for obtaining dye from its leaves. It is an economically important dye plant for obtaining blue dye in the carpet industry of Turkey.

There has been very few studies on *in vitro* propagation of woad and almost all of them emphasize on *in vitro* culture of *I. indigotica* (Peng & Wang, 1994; Hu *et al*., 1999; Chen *et al*., 2000; Zhang *et al.*, 2004). Role of this plant to obtain natural dye could be important, however after recent discoveries of anti-inflammatory potential of lipophilic from its leaf extracts and identification of active principles such as trypanthrin, which are potent inhibitor of prostaglandin and leukotriene (Danz *et al.*, 2001, 2002; Heinemann *et al.*, 2004), its role as an important medicinal plant has increased manifolds. Hamburger (2002) also classified it as important medicinal plant due to the presence of glucobrassicin. Its derivatives play antitumoral role, especially against mammary cancer. This necessitates paying special attention to develop alternative and cheap methods for large scale multiplication of this plant for obtaining these medicinally important compounds in large amount through cheap techniques.

Agar is an important but expensive gelling agent popularly used to solidify most *in vitro* cultures, as it is resistant to metabolism during culture (Handerson & Kinsley, 1988; Ozel *et al.*, 2008). Ozel *et al*. (2008) has emphasized use of isubgol a cheap alternative of agar as it has good gelling ability due to the presence of over 30% mucilage. Moreover it is polysaccharidic and colloidal in nature and resistant to degradative enzymes (Babbar & Jain, 1998; Ozel *et al*., 2008).

Increasing population growth and urbanization have destroyed biological variety gradually (Yucel *et al.*, 2005). It is imperative to conserve ecological properties of natural taxons to obtain biological abundance constatntly using various approaches. Therefore the study was planned to develop an efficient and cheap *in vitro* regeneration methodology for higher shoot regeneration of *I. tinctoria* by comparing the gelling ability of the agar and isubgol gelling agents and find the effects of two on *in vitro* shoot regeneration of the plant.

MATERIALS AND METHODS

Seed source: Dried capsules of *I. tinctoria* were obtained from Associate Professor Dr. Suleyman Kizil, Department

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of Field Crops, Dicle University, Diyarbakir, Turkey. They were surface sterilized using 20% commercial bleach (Ace Turkey-containing 5-6% NaOCl) for 20 min followed by 3 × 3 min rinsing with sterile distilled water. Thereafter, the capsules were gently cut opened using sterilized forceps and surgical lancet to obtain the seeds. These were cultured on MS medium to botain *in vitro* grown seedlings to obtain leaf and hypoctyl explants.

Isolation of explants and culture conditions: Leaf and hypocotyl explants were isolated from *in vitro* germinated 7 d old woad seedlings and cultured on MS (Murashige & Skoog, 1962) medium containing 0.25, 0.50 and 1.00 mg/L BAP-0, 0.25 mg/L NAA (Table I & II) in Magenta GA7 vessels. The regenerated shoots from the best regenerated media solidified with agar and isubgol were rooted on MS medium containing 0.75 mg/L IBA. Each medium was supplemented with 3% sucrose and 0.65% agar (Duchefa, Germany) and 1.5% isubgol (Marahaba Laboratories, Lahore, Pakistan), as suggested by Ozel *et al*. (2008) before autoclaving at 120°C 118 kPa for 20 min. The pH of each medium was adjusted to 5.7-5.8 with 1 N KOH or 1 N HCl before autoclaving. After culturing of explants, they were incubated at 24±2°C for four weeks under cool white fluorescent light provided by Sylvania® Grolux fluroscent tubes (195 µmol photons m^{-2} s^{-1}) with 16 h light photoperiod.

Acclimatization: Rooted shoots (plantlets) were transferred to sterile soil mix containing sand and peat and clay (1:1:1) contained in pots and incubated in growth chambers at room temperature at 70% humidity for 20 days and then transferred to fields. Establishment of the plantlets was determined after 4 weeks of transfer to fields.

Statistical analysis: A split plot experimental design was used to check the effects of two gelling agents-agar and isubgol on shoot regeneration from leaf and hypocotyl. The main treatment consisted of agar and isubgol with two sub treatments (leaf & hypocotyl) with four replications containing 5 explants in each replicate and was repeated twice. Univariate analysis was used to perform analysis of data with SPSS 16 for Windows computer software. Posthoc tests were performed using Duncans Multiple range test. Data given in percentages were subjected to arcsine transformation before statistical analysis (Snedecor & Cochran, 1967).

RESULTS

Effects of agar and isubgol on adventitious shoot regeneraton from leaf explant: Direct shoot regeneration occured from leaf explants both on agar and isubgol containing media without callus formation. The regeneration on leaf explants on both media started from leaf edges on the adaxial side after one week of culture with the development of shoot meristems and shoot initials. These converted to shoots after 13-15 days on MS media contining any concentration of BAP-NAA on both agar and isubgol. With the passage of time the leaf explants on agar containing media began to role and curl, which was very evident after 4 weeks of culture, with 1-2 cm long shoots. Contrarily curling and roling was not observed on any explant regenerated on isubgol containing media. Generally shoot regeneration frequency on agar (60 to 86.67%) was lower compared to isubgol (46.67-100%). Similar trend was observed in number of shoots per explant, which ranged 7.65 to 12.65 on agar and 2.33-17.80 on isubgol. Minimum and maximum number of shoots per explant on agar containing MS medium was recorded on 0.25 mg/L BAP and 1 mg/L BAP-0.25 mg/L NAA (Fig. 1a), respectively on agar. However minimum and maximum number of shoots per explant on isubgol was recorded on MS medium containing 0.25 mg/L BAP and 0.50 mg/L BAP (Fig. 1b), respectively. Moreover it was observed that addition of 0.25 mg/L NAA with any concentration of BAP in agar containing media increased frequency of shoot regeneration and number of shoots per explant. Contrarily, except 0.25 mg/L BAP, all other variants of BAP–NAA behaved variably on isubgol containing media. Shoot length ranged 0.97 to 1.44 cm on agar and 0.40 to 1.47 cm on isubgol. No rooting was recorded on agar with or without 0.25 mg/L NAA. Contrarily moderate rooting in range of 0-33.33% was recorded on isubgol containing media.

Effects of agar and isubgol on adventitious shoot regeneraton hypocotyl explant: Hypocotyl explants also showed direct shoot regeneration both on agar and isubgol containing media without callus formation. Shoot regeneration on hypocotyl explants was little slow compared to leaf explants. Although shoot meristems were first visible after 6-8 days, these developed in to well developed shoots only after 19–20 days of culture on MS mediun contining different concentrations of BAP-NAA. The regeneration on hypocotyl explants on both media started from cut endsof the explants. However agar was inhibitory compared to isubgol with slow growth resulting in smaller shoots. Generally shoot regeneration frequency on agar (80.00 to 100%) was higher compared to isubgol (33.33-86.67%). However number of shoots per explant ranged 8.33 to 19.87 on agar and 5.76-20.55 on isubgol. Minimum number of shoots per explant was recorded on MS medium containing 1 mg/L BAP-0.25 mg/L NAA and 0.25 mg/L BAP-0.25 mg/L NAA on agar and isubgol, respectively. Whereas maximum number of shoots per explant on agar and isubgol was recorded on MS medium containing 1 mg/L BAP (Fig. 1c) and 0.50 mg/L BAP-0.25 mg/L NAA (Fig. 1d), respectively. Negligible to no callusing was recorded on agar and isugol, respectively. However negligible to moderate rooting of 6.67 and 26.67% was recorded on 0.25 mg/L BAP and 0.25 mg/L BAP-0.25 mg/L NAA, respectively.

Rooting: The regenerated shoots from both explants on both gelling agents were rooted on MS medium containing 0,75 mg/L IBA. All plantlets were transferrred to pots after four weeks of culture and acclimatised in the culture room

Table I: Effects of variants of BAP-NAA in agar and isubgol solidified MS medium on shoot regeneration of *Isatis tinctoria* L. subsp. tinctoria L. from leaf explants

BAP (mg/L)	NAA (mg/L)	Frequency (%) of shoot regeneration		Number of shoots per explant		Shoot length (cm)		Frequency (%) of rooting	
		Agar	Isubgol	Agar	Isubgol	Agar	Isubgol	Agar	Isubgol
0.25	0.00	60.00cd	46.67d	7.65cd	2.33d	0.97c	0.40d	0.00a	0.00c
0.25	0.25	80.00ab	86.67b	9.43c	12.73b	1.10b	1.47a	0.00a	33.33a
0.50	0.00	66.67c	100.00a	10.97b	17.80a	1.10b	1.33b	0.00a	33.33a
0.50	0.25	86.67a	80.00b	12.52a	8.81c	1.41a	1.09c	0.00a	20.00b
1.00	0.00	60.00cd	66.67c	8.75cd	7.68c	1.44a	1.08c	0.00a	0.00c
1.00	0.25	86.67a	66.67c	12.65a	4.33cd	0.98c	0.51d	0.00a	0.00c

Table II: Effects of variants of BAP-NAA in agar and isubgol solidified MS medium on shoot regeneration of *Isatis tinctoria* L. subsp. tinctoria L. from hypocotyl explants

BAP (mg/L)	NAA (mg/L)	Frequency (%) of shoot regeneration		Number of shoots per explant		Shoot length (cm)		Frequency (%) of callus regeneration		Frequency (%) of rooting	
		Agar	Isubgol	Agar	Isubgol	Agar	Isubgol	Agar	Isubgol	Agar	Isubgol
0.25	0.00	100.00a	66.67d	12.53c	5.85d	1.43c	0.77d	0.00b	0.00	6.67d	6.67b
0.25	0.25	100.00a	80.00b	17.47b	5.76d	1.90b	0.74d	6.67a	0.00	13.33c	26.67a
0.50	0.00	86.67b	86.67a	9.20d	16.06b	0.58d	0.98cd	0.00b	0.00	0.00e	0.00c
0.50	0.25	80.00bc	73.33 c	19.87a	12.53c	1.23c	1.91a	0.00b	0.00	26.67a	0.00c
1.00	0.00	100.00a	86.67a	17.40b	20.55a	2.50a	1.52b	6.67a	0.00	20.00b	0.00c
1.00	0.25	80.00bc	33.33e	8.33d	11.00c	0.13d	1.13c	0.00b	0.00	0.00e	0.00c

Values within column followed by different small letters are significantly different at the 0.05 level by Duncan's test
Values within row followed by different capital letters are significantly different at the 0.05 level by t test

at ambient conditions of temperature and humidity. These were transferred to fields later on (Fig. 1e & f). They grew to healthy and robust plants, flowered and set seeds after two years.

DISCUSSION

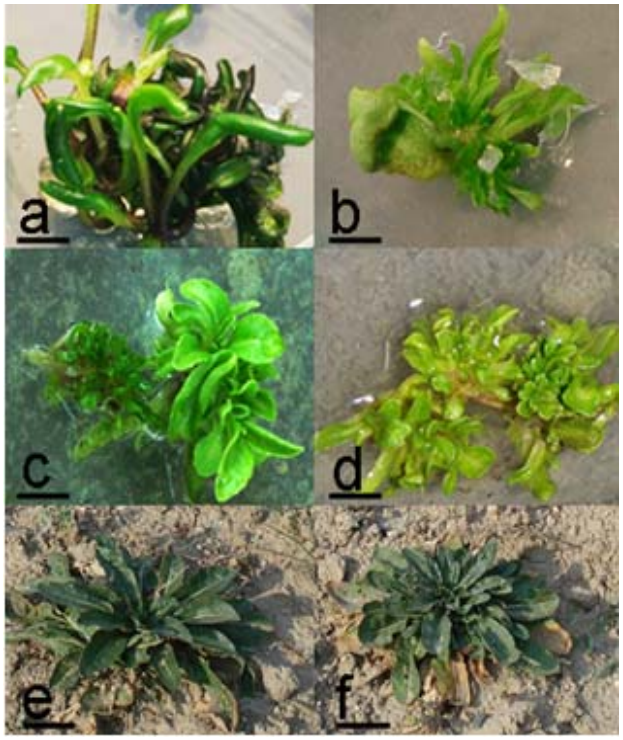
Previous studies report *in vitro* shoot regeneration of *I. indigotica* (Peng & Wang, 1994: Zhang *et al*., 2003, 2004) and *I. aucheri* (Khawar *et al*., 2008) only. The presence of BAP singly or in combinations with NAA influenced shoot regeneration on leaf and hypocotyl explants. Use of BAP-NAA in shoot regeneration has also been emphasised by Khawar *et al.* (2005). Similarly Misra (1996) and Pablo (2002) observed sharp effects type of explant on shoot regeneration. Adventitious shoot regeneration has also been reported in the culture of diplioid *I. indigotica* by Peng and Wang (1994), who emphasized that the MS medium was the best for induction of callus and shoot regeneration from cotyledon explants of *I. indigotica.* Similarly efficient regeneration of tetraploid *I. indigotica* plants from cotyledon and hypocotyl explant was achieved by Zhang *et al.* (2003, 2004). They used BAP-NAA and obtained mean number of 4.2 shoots per explant on hypocotyl explants. However maximum shoot regeneration on agar and isubgol solidified MS medium containing BAP-NAA in two study is much higher. The variation in results could be due to variation in explants species used in two experiments.

No difficulty was recorded in rooting on full strength MS medium supplemented with 0.75 mg/L IBA, in this study. Whereas Zhang *et al.* (2004) reported rooting of *I. indigotica* on ½ strength of MS medium supplemented with 0.5 µm (0.1 mg/L) IBA. However Khawar *et al.* (2008) could not root *I. aucheri* on 0.25 mg/L. IBA, 0.25 mg/L NAA, 0.25 mg/L IBA+0.25 mg/L NAA. They could only root *in vitro* regenerated *I. aucheri* shoots by pulse treatment with 100 mg/L IBA for 5 min.

This experiment compared the effects of agar and isubgol as gelling agents using variants of BAP-NAA for inducing shoot regeneration in the tissue culture experiments using *I. tinctoria.* No genotypic changes were expected in *I. tinctoria* since the experiment made use of same levels of plant growth regulators, nutrient medium, light source and growth temperature. Different types of sugars in agar (D-galactose & 3, 6-anhydrogalactose & agaropectin containing 1, 3- glycosidically linked D-galactose) and isubgol containing xylose, arabinose, galacturonic acid and traces of rhamnose and galactose (Laidlaw & Percival, 1949, 1950). Variable hardness of respective gels due to biochemical and structural differences was expected to affect the molecular diffusion of growth regulators and nutrients through the medium, resulting in quantitative variations in shoot induction in agreement with Ozel *et al.* (2008). As expected the leaf and hypocotyl explants cultured on MS medium containing variants of BAP-NAA using agar and isubgol as gelling agent behaved variably.

Comparing growth parameters, the best adventitious shoot regeneration on leaf and hypocotyl explant using agar gelled medium was obtained on MS medium containing 1 mg/L BAP-0.25 mg/L NAA and 0.5 mg/L BAP-0.25 mg/L NAA, respectively with 12.65 and 19.87 shoots per explant. Contrarily the best adventitious shoot regeneration on leaf and hypocotyl explant using isubgol gelled medium was obtained on MS medium containing 0.5 mg/L BAP with 17.80 shoots per explant and 1mg/L BAP with 20.55 shoots

Fig. 1: Adventitous shoot regeneration from *I. tinctoria* using agar and isubgol as gelling agents (a) shoot regeneration from leaf explant on agar solidified MS medium containing 1 mg/L BAP-0.25 mg/L NAA and (b) isubgol solidified MS medium containing 0.50 mg/L BAP. (c) Shoot regeneration from hypocotyl on agar solidified MS medium containing 0.25 mg/L BAP-0.25 mg/L NAA and (d) isubgol solidified MS medium containing 1 mg/L BAP (e & f) acclimatisation of plants under field conditions, Bar Fig. 1 a,b,c and d = 1 cm, Fig. 1 e and f = 4 cm



per explants, respectively. The results showed that gelling with isubgol was equally suitable for inducing shoot regeneration and induced more number of shoots per explant compared to agar.

The results also showed that isubgol provided better diffusion of media components to the plant tissues, resulting in higher shoot regeneration on two explants due to variable sugars and structural and chemical characteristics of isubgol compared to agar. This could be ascribed to a better contact between explants and the culture medium due to isubgol, which increased the availability of plant growth regulators and other nutrients in the respective media and contributed to enhanced induction of shoot regeneration in line with Ozel *et al.* (2008). Comparatively lower induction of shoots on agar gelled medium may be due to inhibition offered by gelling resulting in lesser diffusion of nutrients through the medium and lesser availability of water to the explants. The results clearly showed that all variations in shoot or root regeneration were mainly due to the types of gelling agents. It was found that plants made use of carbohydrates more efficiently using isubgol compared to agar resulting in more shoots.

In conclusion, the importance of cost effective isubgol as gelling agent in the plant tissue culture has been emphasized. The results suggest that substantial cost reduction with comparatively higher shoot regeneration on isubgol could be easily replaced with agar for *in vitro* propagation of other plants. It will also result in substantial reduction of experimental costs and could be easily used in commercial labs or in countries with sufficiently lower funds for research.

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REFERENCES

Babbar, S.B. and N. Jain, 1998. 'Psyllium' as an alternative gelling agent for plant tissue culture media. *Plant Cell Rep*., 17: 318–322

Chen, W., L.Y. Du, S.X. Cun and F.P. Zhao, 2000. Research on tissue culture from hypocotyl in *I. İndigotica*. *J. Pharm. Pract*., 18: 337–339

Clark, R.J.H., C.J. Cooksey, M.A.M. Daniels and R. Withnall, 1993. Indigo, woad and tyrian purple: important vat dyes from antiquity to the present. *Endeavour,* 17: 191–199

Danz, H., S. Stoyanova, O. Thomet, H.U. Simon, G. Dannhardt, H. Ulbrich and M. Hamburger, 2002. Inhibitory activity of tryptanthrin on prostaglandin and leukotriene synthesis. *Planta Med.,* 68: 875–880

Danz, H., S. Stoyanova, P. Wippich, A. Brattstro and M. Hamburger, 2001. Identification and isolation of the cyclooxygenase-2 inhibitory principle in Isatis tinctoria. *Planta Med.,* 67: 411–416

Hamburger, M., 2002. Isatis tinctoria–from the rediscovery of an ancient medicinal plant towards a novel anti-inflammatory phytopharmaceutical. *Phytochem. Rev*., 1: 333–344

Heinemann, C., S. Schliemann-Willers, C. Oberthu, M. Hamburger and P. Elsner, 2004. Prevention of experimentally induced irritant contact dermatitis by extracts of Isatis tinctoria compared to puretryptanthrin and its impact on UVB-induced erythema. *Planta Med.,* 70: 385–390

Henderson, W.E. and A.M. Kinnersley, 1988. Corn starch as an alternative gelling agent for plant tissue culture. *Plant Cell Tiss. Org. Cult.,* 15: 17–22

Hu, Q., S.B. Andersen and L.N. Hansen, 1999. Plant regeneration from mesophyll protoplasts in *Isatis indigotica*. *Plant Cell Tiss. Org. Cult.,* 55: 155–157

Hurry, J.B., 1930. *The Woad Plant and Its Dye*, Oxford University Press, Oxford, UK

Khawar, K.M., C.A. Ozel, S. Balci, S. Ozcan and O. Arslan, 2005. Efficient shoot regeneration in syrian rue (*Peganum harmala* l.) under in vitro conditions. *Int. J. Agric. Biol.,* 7: 790–793

Khawar, K.M., C.A. Ozel, A. Ulug, I. Sur, E. Kizilates, F. Uzuntas and O. Arslan, 2008. *In vitro* adventitious shoot proliferation of Isatis aucheri Boiss (Turkish woad) from petiole explants. *Res. J. Agric. Biol. Sci.*, 4: 327–330

Laidlaw, R.A. and E.G.V. Percival, 1950. Studies on seed mucilages. Part V. Examination of polysaccharide extracted from the seeds of *Plantago ovata*. Forsk by hot water. *J. Chem. Soc.,* 527–528

Laidlaw, R.A. and E.G.V. Percival, 1949. Studies on seed mucilages. Part III. Examination of polysaccharide extracted from the seeds of *Plantago ovata* Forsk. *J. Chem. Soc.,* 1600–1607

Misra, M., 1996. *In vitro* micropropagation of *Pogostemon cablin* Benth. through callus culture. *Plant Cell Rep.,* 15: 991–994

Murashige, T. and F. Skoog, 1962. Revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.,* 15: 473–497

Ozel, C.A., K.M. Khawar and O. Arslan, 2008. A comparison of the gelling of isubgol, agar and gelrite on *in vitro* shoot regeneration and rooting of variety Samsun of tobacco (*Nicotiana tabacum* L.). *Sci. Hort.*, 117: 174–181

Pablo, E.V., C.H. Andre's, E.V. Ma and P.L. Octavio, 2002. Plant regeneration via organogenesis in Marigold. *Plant Cell Tiss. Organ Cult.*, 69: 279–283

Peng, F. and F.A. Wang, 1994. Study on plant regeneration from cotyledon and hypocotyl of *Isatis indigotica* Fort. *in vitro. J. Hungarican Agric. Coll.*, 20: 45–455

Shu, S.L., 2001. Isatis. In: Taiyan, Z., Z. Lianli, L. Lianli, Y. Guang and I.A. Al-Shehbaz (eds.), *Flora of China,* Vol. 8. Missouri Botanical Garden, Missouri

Snedecor, G.W. and W.C. Cochran, 1967. *Statistical Methods*. The Iowa State University Press, Iowa

Tan, K., 2002. Isatis. *In*: Strid, A. and K. Tan (eds.), *Flora Hellenica,* Vol. 2, pp: 126–127. Ruggell, Gantner Verlag, Germany

Yucel, S., H.H. Ince, H. Mısırdalı and Y. Kara, 2005. Effects of Light on the Germination of *Isatis demiriziana* Misirdali and *Isatis constricta* Davis's Seeds. *Int. J. Agric. Biol.,* 7: 150–151

Zhang, L., G. Kai, T. Xu, Y. Pi, X. Sun and K. Tang, 2004. Efficient regeneration of tetraploid *Isatis indigotica* plants via adventitious organogenesis from hypocotyl explants. *Biol. Plant.*, 48: 121–124

Zhang, L., T. Xu, X. Sun, H. Zhang, T. Tang and K. Tang, 2003. Factors influencing shoot regeneration from cotyledons of tetraploid *Isatis indigotica* Fort. *In vitro Cell. Dev. Biol. Plant.,* 39: 459–462

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