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Adventitious shoot regeneration from leaf, stem and root explants of commercial cultivars of *Gentiana*

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Abstract

Several culture conditions were examined for promoting efficient plant regeneration from explants of *Gentiana*. Adventitious shoot regeneration from leaf explants of cv. WSP-3 was very superior on MS medium, compared to B5 medium, supplemented with four cytokinins (TDZ, 4PU-30, BA and zeatin). An auxin / cytokinin combination was required for regeneration. TDZ was the most effective cytokinin, while NAA was more effective than IAA or 2,4-D. Optimum conditions for regeneration from explants (leaf, stem and root) of cv. WSP-3, evaluated in terms of regeneration frequency and number of regenerated shoots per explant, were TDZ and NAA in combination, 5 - 10 mg/l and 0.1 mg/l for leaf and stem explants, and 10 mg/l and 1 mg/l for root explants, respectively. Application of these conditions to eight other commercial cultivars resulted in 30 - 100% regeneration from leaf explants. The number of chromosomes in each of ten regenerated plants of each cultivar was diploid, 2n=26. Shoots regenerated *in vitro* were rooted in phytohormone-free medium and transferred to soil.

Key words: *Gentiana*, plant regeneration, TDZ

Abbreviations: MS medium, Murashige and Skoog's medium (Murashige and Skoog 1962); B5 medium, Gamborg B5 medium (Gamborg et al. 1968); BA, 6-benzylaminopurine; TDZ, N-phenyl-N'-1,2,3-thiadiazol-5-yl urea; 4PU-30; N-(2-chloro-4-pyridyl)-N'-phenylurea; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid; NAA, 1-naphthaleneacetic acid

Introduction

Gentiana spp. are herbaceous perennials of ornamental importance in Japan. Adventitious shoot regeneration in *Gentiana* has been reported only from protoplasts (Takahata and Jomori 1989), but the frequency is quite low. Attempts to induce adventitious shoot regeneration from leaf explants of *G. lutea* and *G. punctata* have not met with success (Skrzypczak et al. 1993). The present study was thus conducted to induce plant regeneration from leaf, stem and root explants of cv. WSP-3. Optimal conditions for regeneration from nine commercial cultivars of *Gentiana* are also presented.

Materials and methods

Plant materials: Seeds of five *Gentiana triflora* cultivars (F1 hybrid) Homoi, Ïhatovo, Iwate, Iwate-otome and Maciry; an interspecific hybrid cultivar (F1 hybrid, *G. triflora* x *G. scabra*) Albireo; and three *in vitro* shoot cultures of interspecific hybrid cultivars (clonal variety, *G. triflora* x *G. scabra*) H-3, Polarno white and WSP-3, were obtained from Iwate Horticultural Experiment Station. The *in vitro* shoot culture of cv. WSP-3 was used mainly to establish optimal conditions for adventitious shoot regeneration. Seeds were surface-disinfected with 1% sodium hypochlorite solution for 5 min followed by two rinsings in sterilized distilled water. The disinfected seeds were germinated on MS medium supplemented with 30 g/l sucrose and 2 g/l gellan gum. All axillary buds were cultured on the same medium.

Plantlets from the seedlings or *in vitro* shoot cultures were routinely subcultured at 20℃ for 16 h daytime under fluorescent lamps (50 μ mol m^{-2} s^{-1}). Subcultures were made once every two months by transferring terminal and lateral cuttings into the same medium.

Leaf (approximately 5 mm square), stem and root explants (approximately 5 mm in length) were aseptically excised from *in vitro* shoot cultures for use in the regeneration experiments.

Media and culture conditions: Basic MS and B5 media supplemented with 30 g/l sucrose and various plant growth regulators at different concentrations were used. The media were solidified with 2 g/l gellan gum and pH was adjusted to 5.7-5.8 with 1 N NaOH prior to autoclaving. All cultures were incubated under the conditions specified above. Leaf explants of cv. WSP-3 were cultured on MS and B5 media, both supplemented with four cytokinins (TDZ, 4PU-30, BA and zeatin; 0 to 10 mg/l), along with NAA (0 to 1 mg/l).

To determine the optimum concentration of TDZ and effects of auxins introduced in combination with TDZ on regeneration, leaf explants of cv. WSP-3 were cultured on MS medium with TDZ (0 to 20 mg/l) and three auxins (NAA, IAA and 2,4-D; 0 to 1 mg/l) present at different concentrations.

Stem and root explants of cv. WSP-3 were cultured on MS medium containing different concentrations of TDZ (0 to 20 mg/l) and NAA (0 to 1 mg/l).

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Leaf explants of nine commercial cultivars were cultured on MS medium with TDZ (0 to 20 mg/l) in combination with NAA (0 to 1 mg/l).

Regenerated shoots were transferred to MS medium containing 30 g/l sucrose and 2 g/l gellan gum for rooting. The regenerated plants with a well-established root system were carefully rinsed to remove gellan gum and transferred to pots containing vermiculite. Plants in the pots were then transferred to a greenhouse following completion of acclimatization.

Chromosome count: Root tips were excised from *in vitro* grown plantlets after two weeks and pretreated with 2 mM 8-hydroxyquinoline at 20℃ for 4 to 5 h. This was followed by fixation in 3:1 absolute alcohol : glacial acetic acid for at least 24 h and hydrolysis with 1 N HCl at 60℃ for 5 min. The tips were then rinsed with distilled water and stained with 2% aceto-orcein. The chromosomes were counted for at least five well-spread root tip cells from at least two separate roots.

Results and Discussion

Effects of basal medium and plant growth regulators: Adventitious shoot regeneration from leaf explants of cv. WSP-3 was more frequent on MS medium than

Table 1. Effects of media (MS and B5) and cytokinins (TDZ, 4PU-30, BA and Zeatin) in combination with NAA on regeneration of adventitious shoots from leaf explants of cv. WSP-3. Cultures were scored at eight weeks. Twenty explants were used for each experiment.

Cytokinin (mg/l)		MS medium NAA(mg/l)			B5 medium NAA(mg/l)		
		0	0.1	1	0	0.1	1
	0	0	0	0	0	0	0
TDZ	1	0	95	30	0	0	0
	10	0	100	55	0	0	0
4PU-30	1	0	60	70	0	40	30
	10	0	20	10	0	0	0
BA	1	0	20	30	0	0	0
	10	0	70	5	0	0	0
Zeatin	1	0	10	0	0	10	0
	10	0	10	50	0	0	0

Table 2. Effects of TDZ in combination with auxins (NAA, IAA and 2,4-D) on adventitious shoot regeneration from leaf explants of cv. WSP-3. Cultures were scored at eight weeks. Twenty explants were used for each experiment.

TDZ (mg/l)	Auxins (mg/l)	NAA		IAA		2,4-D	
		Adventitious shoot regeneration (%)	No. of adventitious shoot / explant	Adventitious shoot regeneration (%)	No. of adventitious shoot / explant	Adventitious shoot regeneration (%)	No. of adventitious shoot / explant
0	0	0	0	0	0	0	0
	0.01	0	0	0	0	0	0
	0.1	0	0	0	0	0	0
	0.5	0	0	0	0	0	0
	1	0	0	0	0	0	0
1	0	0	0	0	0	0	0
	0.01	60	2.0 ± 0.8*	5	3.0 ± 0	5	3.0 ± 0
	0.1	95	8.6 ± 2.7	80	7.3 ± 3.5	65	4.3 ± 2.2
	0.5	55	5.0 ± 3.3	70	8.0 ± 4.4	25	3.4 ± 1.5
	1	30	1.6 ± 0.5	60	6.8 ± 3.8	0	0
5	0	0	0	0	0	0	0
	0.01	70	8.1 ± 2.8	25	6.8 ± 4.4	5	3.0 ± 0
	0.1	100	9.0 ± 4.7	90	7.0 ± 3.7	65	8.6 ± 6.1
	0.5	85	5.2 ± 2.5	80	7.3 ± 2.8	35	2.5 ± 1.0
	1	45	1.9 ± 0.8	95	11.4 ± 4.6	5	1.0 ± 0
10	0	0	0	0	0	0	0
	0.01	20	2.5 ± 1.5	20	6.0 ± 2.0	0	0
	0.1	100	10.3 ± 4.9	65	3.7 ± 2.8	50	6.5 ± 1.9
	0.5	95	5.3 ± 3.4	70	10.6 ± 3.3	20	1.8 ± 0.8
	1	55	3.3 ± 2.3	90	8.9 ± 4.0	0	0
20	0	0	0	0	0	0	0
	0.01	20	2.0 ± 0	0	0	0	0
	0.1	100	6.4 ± 2.9	30	5.5 ± 3.0	25	2.4 ± 1.5
	0.5	95	9.7 ± 3.8	85	8.9 ± 4.3	15	3.0 ± 1.4
	1	55	5.6 ± 2.6	100	10.1 ± 4.2	0	0

* Mean ± standard deviation shown.

Table 3. Effects of TDZ in combination with NAA on adventitious shoot regeneration from stem and root explants of cv. WSP-3. Cultures were scored at eight weeks. Twenty explants were used for each experiment.

TDZ (mg/l)	NAA (mg/l)	Stem explants		Root explants	
		Adventitious shoot regeneration (%)	No. of adventitious shoot / explant	Adventitious shoot regeneration (%)	No. of adventitious shoot / explant
0	0	0	0	0	0
	0.01	0	0	0	0
	0.1	0	0	0	0
	0.5	0	0	0	0
	1	0	0	0	0
1	0	0	0	0	0
	0.01	100	3.0 ± 1.7*	30	1.7 ± 0.9
	0.1	80	6.0 ± 1.2	50	1.4 ± 0.5
	0.5	100	3.8 ± 1.0	30	1.7 ± 0.5
	1	100	2.3 ± 1.3	0	0
5	0	0	0	0	0
	0.01	80	3.3 ± 2.3	30	1.7 ± 0.9
	0.1	100	8.7 ± 2.1	60	2.3 ± 1.1
	0.5	80	9.5 ± 4.2	60	3.5 ± 1.5
	1	100	4.7 ± 0.5	20	4.0 ± 0
10	0	0	0	0	0
	0.01	60	5.3 ± 0.5	50	1.8 ± 1.0
	0.1	100	9.3 ± 2.6	80	3.4 ± 2.7
	0.5	60	4.3 ± 2.4	80	4.0 ± 2.1
	1	20	1.0 ± 0	100	4.8 ± 2.5
20	0	0	0	0	0
	0.01	0	0	10	1.0 ± 0
	0.1	60	14.0 ± 8.3	20	1.0 ± 0
	0.5	60	7.0 ± 2.8	60	3.8 ± 1.3
	1	60	4.3 ± 2.1	50	2.8 ± 0.8

* Mean ± standard deviation shown.

B5 medium in all cases (Table 1). TDZ (1 - 10 mg/l) in combination with NAA (0.1 mg/l) proved to be optimal for regeneration, followed by 4PU-30, BA and zeatin. The presence of an auxin was required, as no regeneration was obtained with cytokinin alone.

In order to establish optimum conditions for regeneration, TDZ was used in combination with three auxins. NAA was the most effective for regeneration from leaf explants at 0.1 mg/l when combined with 5 - 10 mg/l TDZ (regeneration frequency, 100%; Table 2, Fig. 1A). Although 100% regeneration frequency was also attained on MS medium containing 20 mg/l TDZ along with 0.1 mg/l NAA and 1 mg/l IAA, some of the shoots were vitreous, which hindered subsequent rooting and acclimation. 2,4-D was the least suitable.

Regeneration from stem and root explants: Regeneration from stem and root explants was greatest at 5 - 10 mg/l TDZ in combination with 0.1 mg/l NAA, and at 10 mg/l TDZ in combination with 1 mg/l NAA, respectively (Table 3). For root explants, only five adventitious shoots per explant were formed which is less than under the optimum conditions for leaf and stem explants. The best conditions for maximum regenerated shoots per explant are shown in Tables 2 and 3. Leaf and stem explants were found to be most suitable for shoot regeneration.

Regeneration from other cultivars: Adventitious shoot regeneration from the leaf explants of nine commercial cultivars on MS medium containing TDZ and NAA was assessed (Table 4). Cultivars Albireo, H3, Polarno white and WSP-3 that were bred from *G. scabra* as pollen parents showed higher regeneration (80 and 100%), whereas *G. triflora* cultivars Homoi, Ïhatovo, Iwate, Iwate-otome and Maciry showed less regeneration (30 to 75 %). A comparison of mean numbers of regenerated shoots indicated cv. WSP-3 and Polarno white to have the highest value for this parameter (8 - 10 shoots per explant).

Recently, TDZ has been applied to herbaceous plants such as carnation (Frey and Janick 1991, Nugent et al. 1991), flax (Bretagne et al. 1994) and peanut (Kanyand et al. 1994). TDZ has been shown to be effective also for micropropagation and adventitious shoot regeneration from woody plants (Huetteman and Preece 1993). In *Picea glauca*, TDZ was as or more effective for regeneration than BA, kinetin and zeatin (Ellis et al. 1991). Further, TDZ may have auxin activity (Lu

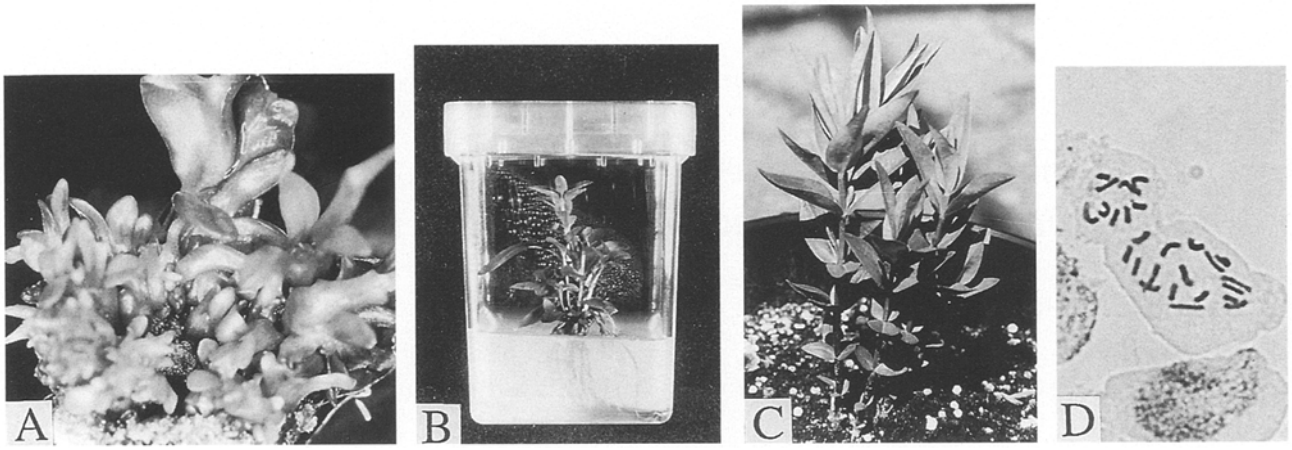

Fig. 1. Regeneration of plants from leaf explants of the *Gentiana* cv. WSP-3. (A) Adventitious shoots regenerated from leaf explants at eight weeks of culture on MS medium supplemented with 10 mg/l TDZ and 0.1 mg/l NAA. (B) Development of adventitious roots on regenerated shoots in plant growth regulator free MS medium at four weeks of culture. (C) Three-month-old regenerated plants placed in soil in pots. (D) Chromosomes in root tip cells of regenerated plants (2n=26).

1993). TDZ induced shoot regeneration to essentially the same degree, with or without NAA, in carnation (Nugent et al. 1991). In this study, NAA was required for effective regeneration using TDZ.

Increase in the incidence of vitrification with the concentration of TDZ (Briggs et al. 1988a, 1988b, Gribaudo and Fronda 1991) has been reported. We did not detect any vitrification of regenerated shoots of *Gentiana* at 10 mg/l TDZ. Some abnormalities including short, compact and fasciated shoots associated with the use of TDZ (Huetteman and Preece 1993, Lu 1993) have been reported, but were not noted in this study on MS medium containing TDZ in nine cultivars of *Gentiana*.

Table 4. Adventitious shoot regeneration from leaf explants of nine cultivars of *Gentiana* spp. in culture on MS medium supplemented with TDZ and NAA at different concentrations. Cultures were scored at eight weeks. Twenty explants were used for each experiment.

Cultivar	Regeneration (%)	No. of regenerated shoots per explant	Culture condition TDZ - NAA (mg/l)
Albireo	80	3.4 ± 1.1*	10 - 1
Homoi	40	1.8 ± 0.5	5 - 0.1
H3	100	3.2 ± 0.9	10 - 1
Ïhatovo	60	2.9 ± 1.2	10 - 0.1
Iwate	50	2.4 ± 0.6	5 - 1
Iwate-otome	30	2.6 ± 0.6	10 - 1
Maciry	75	2.4 ± 0.7	5 - 1
Polarno white	100	8.5 ± 2.3	10 - 0.1
WSP3	100	10.3 ± 4.9	10 - 0.1

* Mean ± standard deviation shown.

Morphological and chromosormal observations: Shoots produced in culture were easily rooted in phytohormone-free medium in four weeks, and then acclimatized (Fig. 1B). Each of twenty regenerated plants grown in a greenhouse were phenotypically normal with respect to leaf shape and growth features during early growth stages (Fig. 1C). The number of chromosomes was counted in the root tip cells of ten regenerated plants of each cultivar, and was found to be 2n=26 (Fig.1D), which corresponds to the diploid number for the species.

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