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Protoplasts to plants of Gentianaceae. Regeneration of lisianthus (*Eustoma grandiflorum*) is affected by calcium ion preconditioning, osmolality and pH of the culture media

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Abstract

A reliable method has been developed for regeneration of whole plants from isolated protoplasts of five cultivars of lisianthus, *Eustoma grandiflorum* (Griseb.) Schinners (Gentianaceae). Protoplasts were isolated from either cotyledons or leaves and cultured in agarose beads surrounded by liquid V-KM media containing 5.37 μM 1-naphthyleneacetic acid (NAA) and 2.28 μM zeatin. When microcalli were approximately 1 mm in diameter, the agarose beads were transferred to shoot regeneration media containing 0.1 μM indolebutyric acid (IBA) and 4.44 μM 6-benzylaminopurine (BAP). Shoots were produced from the calli during several sub-culture periods. Protoplast viability and the subsequent regeneration of plants were dependent on calcium levels and growth regulator presence in the *in vitro* seed germination media, on the osmolality of the protoplast purification solution, and osmolality increase and pH of the culture media. Shoots were rooted in Murashige & Skoog (1962) media containing 5.71 μM indole-3-acetic acid (IAA). Plantlets derived from protoplasts of five lisianthus cultivars (Fresh White, Hakusen, Miss Lilac, Fresh Purple and Doremi Wine Red) have been successfully transferred to the glasshouse.

Abbreviations: B5 – Gamborg et al. (1968), BAP – 6-benzylaminopurine, IAA – indole-3-acetic acid, IBA – indolebutyric acid, GA_3 – gibberellic acid, mOsm – (negative) milli Osmoles per kilogram water, MES – 2[N-morpholino]ethane sulfonic acid, MS – Murashige & Skoog (1962), NAA – α-naphthaleneacetic acid, pfd – photonfluence density, V-KM – Binding & Nehls (1977)

Introduction

Lisianthus (*Eustoma grandiflorum* Griseb.), a member of the family Gentianaceae, is an annual endemic to the prairies of the south mid-western United States. In cultivated varieties, flower colour in both single and double forms ranges from white through pink to purple. Lisianthus is commercially cultivated as a cut flower and as a potted flowering plant. The cut inflorescences typically have a vase life of 3 to 6 weeks (Dennis et al. 1989).

Studies on extending the range of flower colours by *in vitro* direct gene uptake and protoplast fusion depend on first developing a successful protoplast to plant regeneration system. During initial attempts to isolate protoplasts from shoot tips grown on half-strength MS media, the protoplast membranes were disrupted in the wash solution. A few shoots regenerated,

however, when the shoot tips had been grown on full strength MS containing growth regulators. Consequently, to improve the level of regeneration from lisianthus protoplasts, we investigated whether the presence of plant growth regulators or the level of macronutrients would affect the yield and viability of protoplasts, and therefore plantlet regeneration.

Materials and methods

Shoot regeneration from protoplasts

Growth of source-plant material
The germination medium consisted of MS (Murashige & Skoog 1962) macro- and micronutrients and contained B5 vitamins, 2% sucrose, 0.23 μM IBA, 1.33 μM BAP, and 0.29 μM GA_3. Agar was added at 0.8% and pH adjusted to 5.8 prior to autoclaving at 121°C for 15 min. IAA or GA_3 were filter sterilised and added into the autoclaved media. Seeds of lisianthus cultivars Fresh White, Hakusen, Miss Lilac, Fresh Purple and Doremi Wine Red were obtained from Dai-ichi Seed Co. Ltd, Japan. These were surface sterilized with a 0.6% sodium hypochlorite solution and rinsed in sterile distilled water three times before mixing with sterile 0.2% water-agar. The mixture was pipetted onto germination media. Seeds were germinated at 25°C under 40 μmol m^{-2} s^{-1} ppf (Philips White) with 16-h photoperiod.

Protoplast isolation and purification
Excised shoot tips were digested overnight at 22°C in an enzyme mixture containing 1.0% Cellulysin (Calbiochem), 0.3% 'Onozuka' Macerozyme R-10 (Yakult Honsha), and 0.05% Pectolyase Y-23 (Seishin Pharmaceuticals), supplemented with 0.5 M sorbitol and 5 mM $Ca(NO_3)_2$. Protoplasts were sieved through 35 μm steel mesh, and pelleted by centrifugation ($100 \times g$ for 5 min). The protoplasts were resuspended in a wash solution of 1/10 strength V-KM macronutrients (Binding 1974) containing 2.69 mM NaCl (550 mOsm), and repelletted at $80 \times g$ for 5 min.

Culture of protoplasts
The protoplasts were embedded in 2% agarose (Sigma Type VII) as 'streaky plate lenses' (Binding et al. 1988). They were surrounded by liquid V-KM culture medium at 500 mOsm (Binding & Nehls 1977) containing 5.37 μM NAA and 2.28 μM zeatin. The protoplasts were incubated in darkness at 22°C. The liquid medium was changed after the first day, and then at weekly intervals.

Shoot regeneration
For shoot regeneration, agarose beads containing microcallus of approximately 100 cells were transferred to MS media containing 0.098 μM IBA and 4.44 μM BAP solidified with 0.75% Difco Noble agar. The beads were incubated under 40 μmol m^{-2} s^{-1} pfd with 16-h photoperiod. After one week the lenses were spread to separate individual microcalli and were then replated on fresh medium. When shoots developed with five or more leaves they were removed from the callus and transferred to MS medium containing 0.23 μM IBA, 1.33 μM BAP, and 0.29 μM GA_3 for growth. The callus was replated for further shoot regeneration. Root formation was initiated by placing shoots on MS medium containing 5.7 μM IAA for one week. The plantlets were then transferred onto half-strength MS medium for root development. Rooted plantlets were acclimatised to glasshouse conditions under a mist propagation tent.

Effects of macronutrients and/or growth regulators

Growth of source-plant material
To test the effects of macronutrients and growth regulators on protoplast viability and regeneration, surface sterilised seeds of lisianthus cultivars were pipetted with sterile 0.2% water-agar onto different modifications of MS media (Table 2).

Protoplast culture studies
Four-week-old cotyledons were excised and the protoplasts isolated as described above. The wash solution was at either the osmolality of 550 mOsm (normally used) or, a higher osmolality of 600 mOsm (0.31 M NaCl).

Protoplast yield was determined using a haemocytometer and viability determined by fluorescein diacetate (Widholm 1972). The osmolality of the solutions was measured on a Fiske OS osmometer (Fiske Associates, MA, USA). The osmolality of the V-KM media was measured at 500 mOsm. A higher, 600 mOsm level medium, was made with additional sorbitol. After one day the osmolality was decreased by three steps: 1:1, 1:2 and 0:1 ratios of 600:500 mOsm media at days one, three and seven after protoplast isolation. The medium was then changed weekly.

Osmolality and pH effects on protoplast culture

To further test the effects of osmolality and pH, protoplasts were plated in V-KM media at either 500 or 600 mOsm at pH 5.8 with or without buffering (3 mM MES).

Results

Shoot regeneration from protoplasts

Viable protoplasts re-formed cell walls, and cells divided three to four days after plating. Cell clusters, arising from single protoplasts, remained independent for up to seven days after isolation, after which time they coalesced, and individual microcalli became indistinguishable within the bead. However, nodules of callus formed and these could be separated after transfer to the agar-solidified media. Leaf primordia were observed (Fig. 1) between 77 and 105 days after protoplasts were isolated. The regeneration time was dependent on the cultivar and the level of plant growth regulators in the source-plant growth media. The cultivar Fresh White had a greater regeneration capability than the other cultivars tested. All five cultivars varied in their ability to release viable protoplasts and to regenerate plants, depending on the culture conditions. In total more than 400 plants were regenerated from the five lisianthus cultivars, successfully transferred into soil, and grown in the glasshouse.

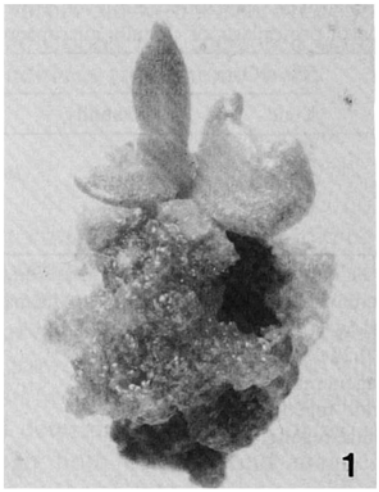

Fig. 1. Shoot regeneration on MS media containing 0.098 μM IBA and 4.44 μM BAP from lisianthus cultivar Fresh White protoplast-derived callus.

Effect of macronutrients and growth regulators

Shoot tips grown on full MS (Table 1) released $5.4–5.7 \times 10^5$ protoplasts per gram fresh weight, with high viability according to FDA testing in both wash solutions. Fewer protoplasts were released from source-plants grown on half-strength MS, and although they were viable in the enzyme mix, they burst in the 550 mOsm wash solution. When the 600 mOsm wash solution was used, highly viable protoplasts were obtained which re-formed cells, divided and regenerated plants. Shoot tips cultured in the presence of growth regulators released more protoplasts ($1.6–1.7 \times 10^6$ protoplasts per gram). Similar effects of osmolality were noted as above, but the presence of plant growth regulators reduced plant regeneration.

Viability and the regeneration ability of protoplasts isolated from seedlings of cv Fresh White were affected by a number of variables (Table 2). For seedlings grown on media 1–6, the highest regenerative potential of protoplasts occurred when the level of $CaCl_2$ was 2.98 mM, irrespective of the level of $MgSO_4$, or the osmolality of the wash solution. Low $CaCl_2$ (1.49 mM) was detrimental in either full or half-strength MS when the protoplasts were treated with 550 mOsm wash. This effect was overcome

Table 1. Effect of MS macronutrient level, growth regulator presence, and osmolality of the wash solution on the viability and regeneration of protoplasts of clonally micropropagated *Eustoma grandiflorum* cv. Fresh White.

Media[a]	550 mOsm			600 mOsm		
	Yield	Viability	Regeneration[b]	Yield	Viability	Regeneration
1	5.7×10^5	88%	+ + +	5.4×10^5	90%	+ + +
2	3.5×10^5	16%	burst	4.0×10^5	96%	+ +
3	1.6×10^6	92%	+	1.5×10^6	88%	+
4	1.7×10^6	10%	burst	1.7×10^6	92%	+

[a] Basal media contained MS micronutrients, iron, B5 vitamins, 3% sucrose, 0.8% agar (Davis) at pH 5.8 and macronutrients as: 1. MS macronutrients at full strength, 2. MS macronutrients at half strength, 3. MS macronutrients at full strength containing 0.23 μM IBA, 1.33 μM BAP, and 0.29 μM GA_3 and 4. MS macronutrients at half strength containing 0.23 μM IBA, 1.33 μM BAP, and 0.29 μM GA_3.

[b] + poor regeneration.
+ + moderate regeneration.
+ + + optimal regeneration.

in the half-strength MS, however, when the protoplasts were washed in 600 mOsm solution. The higher $CaCl_2$ concentration (5.89 mM) always gave a lower regenerative potential irrespective of the MS concentration or the osmolality of the wash solution. The addition of plant growth regulators altered the growth habit, creating rosettes of leaves rather than plants with elongated stems. Callus formed at the interface between the seedling and the medium, preventing root elongation. Vitrification was observed on all plants except those grown with 2.98 mM $CaCl_2$; only non-vitrified seedlings produced protoplasts which were able to regenerate.

Changing the levels of $MgSO_4$ (media 13–14, Table 2) did not appear to affect protoplast viability. When calcium or magnesium was reduced to half the normal level in full-strength

Table 2. Effect of seedling growth, media and osmolalities of the wash solution on the viability and regeneration of protoplasts of *Eustoma grandiflorum* cv. Fresh White.

Media	Media concentrations				Seedling plant growth[2]	Wash solutions			
	$CaCl_2$ mM	$MgSO_4$ mM	Other macros	Growth regulators[1]		550 mOsm viability	Growth[3] stage	600 mOsm viability %	Growth stage[3]
1	1.49	1.5	full	–	+, el, r	0	0	0	0
2	2.98	1.5	full	–	+ +, el, r	88	d, m, r	87	d, m, r
3	5.89	1.5	full	–	+ +, el, r	90	d, m	84	d
4	1.49	0.75	half	–	+ +, el, r	28	b	92	d, m, r
5	2.98	0.75	half	–	+ + +, el, r	92	d, m, r	92	d, m, r
6	5.89	0.75	half	–	+, ros, r	76	d, m	64	d
7	1.49	1.5	full	+	v, c	12	0	25	0
8	2.98	1.5	full	+	+ +, ros, c	93	d, m, r	84	d, m, r
9	5.89	1.5	full	+	+ +, ros, v, c	84	d	90	d
10	1.49	0.75	half	+	+, ros, v, c	12	b	96	0
11	2.98	0.75	half	+	+ + +, el, c	78	d, m, r	82	d, m, r
12	5.89	0.75	half	+	v, c	0	0	0	0
13	2.98	0.75	full	–	low germ	0	0	0	0
14	2.98	3.0	full	–	+ +, el, r	75	d, m	72	d, m

[1] Plant growth regulators were absent (–) or when present (+) comprised 0.23 μM IBA, 1.33 μM BAP, and 0.29 μM GA_3.
[2] Donor plant growth: + = poor growth, + + = moderate growth, + + + = optimal growth; el = elongated shoot growth, ros = rosette leaf formation without extended shoot growth, r = roots present, c = callus present, v = vitrified, low germ = low germination rate.
[3] Growth Stage: 0 = non-viable protoplasts, b = protoplasts burst in the wash solution, d = cell division, m = microcallus formation, r = plantlet regeneration.

Table 3. Time to cell division induced by pH and/or osmolality of plating media for cv. Fresh White.[1]

Treatment	mOsm	pH	Time to first division (days)
1	600	5.8–5.1	4
2	500	5.8–5.1	3
3	600–500	5.8–5.1	2
4	600	5.8[2]	7
5	500	5.8[2]	6
6	600–500	5.8[2]	4

[1] Protoplasts were isolated from seedlings grown on full MS (Medium 2, Table 2), isolated at 600 mOsm and the V-KM adjusted as indicated. All experiments were repeated at least three times.
[2] V-KM media buffered with 3 mM MES.

MS (media 1 and 13, Table 2) plant growth was poor, resulting in poor protoplast viability and yield.

Osmolality and pH effects on protoplast culture

Alteration of the osmolality or pH or both in the V-KM medium, one day after the protoplasts had been embedded, influenced the onset of cell division. When the protoplasts were plated at 600 mOsm (Table 3, treatment 1), cell division was first observed four days after embedding. At 500 mOsm, cell division occurred earlier and with a greater frequency (treatment 2). When the osmolality was increased after the first day, cells divided on the second day (treatment 3). The pH of the V-KM media altered from 5.8 to 5.1 in highly viable cultures; non-viable cultures altered to a pH of 4.5. When the plating media was buffered to pH 5.8 with 3 mM MES, the time to first cell divisions was increased under all osmotic conditions (treatments 4–6).

Discussion

The most critical factor in determining the success of protoplast regeneration from lisianthus was the preconditioning of the source-plant material. Not only did the source-plant media affect plantlet growth, it also influenced whether the protoplasts would survive isolation and purification, and determined their rate of regeneration.

Although both calcium and magnesium are important in the stability and permeability of the plasma membrane (Clarkson & Hanson 1980), it appears that calcium plays a more critical role in plasma membrane stability of lisianthus protoplasts. If the $CaCl_2$ level was 2.98 mM, regardless of the other macronutrient levels, the protoplasts would not burst at 550 mOsm. However at either 1.49 or 5.89 mM $CaCl_2$ seedling growth was adversely affected (Fig. 2). Here the protoplasts were either susceptible to bursting in the wash solution or produced protoplasts with a poor regeneration potential. This concurs with the results of Chang & Loescher (1991) who showed that calcium pretreatment increased protoplast yield and plantlet regeneration from *Solanum tuberosum*. The calcium-induced effects on lisianthus were specific; alteration of the $MgSO_4$ levels (0.75, 1.5 or 3.0 mM) in the seedling growth media resulted in poor plant

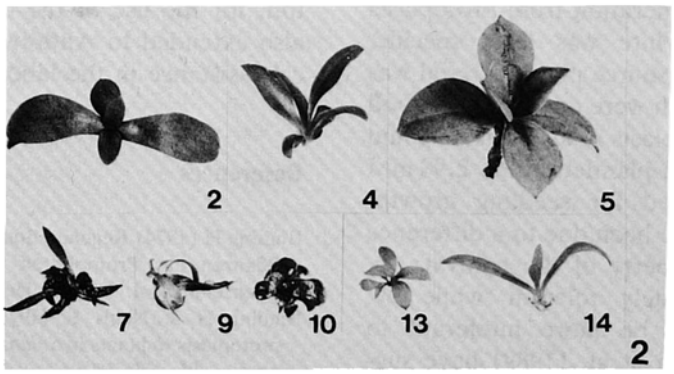

Fig. 2. The effects of different macronutrient levels on plant growth of lisianthus. Numbers beside each plant indicate the media used for seedling growth. These media are defined in Table 2.

growth, but did not reduce protoplast release or viability.

Plant growth regulators have been found to affect passive and active mineral uptake processes, for instance auxin may be involved in localised calcium movements (Raven & Rubery 1982). The formation of basal callus on the donor plant cultures in the presence of plant growth regulators may have interfered with mineral permeability. No such repression of mineral permeability is present in a normal root system.

Vitrification, observed when the source-plant material was grown on either 1.49 or 5.89 mM $CaCl_2$, prevented successful protoplast isolation and therefore plantlet regeneration. Vitrification did not occur when 2.98 mM $CaCl_2$ was incorporated in the media, indicating a possible role for calcium in preventing vitrification.

Seedlings grown on media containing 5.89 mM $CaCl_2$ (Table 2, media 3, 6 and 9) yielded highly viable protoplasts but these did not form microcalli or regenerate plantlets; seedlings grown on media containing plant growth regulators (Table 1, media 3 and 4; Table 2, medium 10) also failed to form microcallus. These conditions, i.e. high calcium concentration or plant growth regulator presence, or both, cause stress to the donor plants before protoplasts are isolated. Smith et al. (1989), suggest that stressed protoplasts may not be visualised by the fluorescein diacetate viability stain, but cells derived from stressed protoplasts will not undergo sustained cell division.

Variation was noted in the response of different cultivars to the treatment conditions. Cv. Hakusen was the most specific in its requirements for plantlet regeneration from protoplasts whereas cv. Fresh White was least specific, regenerating whenever source plant material was grown on medium which were conducive to good seedling growth. Protoplasts of all cultivars could be regenerated to form plantlets when 2.98 mM $CaCl_2$ was incorporated for seedling growth. This variation may have been due to a difference in the plasmolysis property of the cultivar; cv. Fresh White was widely tolerant while cv. Hakusen appeared to be more intolerant to osmotic changes. Smith et al. (1989) have suggested a difference in adherence of the plasmalemma to the cell wall in the source-plant material can affect the degree of plasmolysis, and therefore the regeneration ability of the isolated protoplasts.

The reduction in osmolality and pH in the plating media together appear to act as a signal for the initiation of cell division. The alteration of osmolality shortly after the isolation of protoplasts caused cell division to start earlier, and increased the rate of cell division. This result concurred with that of Burgess (1983) who found that lowering of osmolality played an important role in cell division. The pH in the lisianthus protoplast cultures stabilised at 5.1 in viable, dividing cultures. Buffering of the medium delayed cell division and reduced the frequency.

The detrimental effect of plasmolysis of lisianthus protoplasts at 550 mOsm was overcome by growing seedlings at the higher calcium ion level, suggesting an interaction between protoplast stability and macronutrient content of the media used to culture the protoplasts. The importance of the initial medium used for the source-plant material and the increasing of the osmolality extends beyond protoplast isolation as it also affects the regeneration capability of the lisianthus protoplasts. This is the first report of successful plant regeneration from protoplasts from the family Gentianaceae.

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