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BRIEF COMMUNICATION

Clonal propagation of *Zephyranthes grandiflora* using bulbs as explants

M. GANGOPADHYAY[1]*, D. CHAKRABORTY[2], S. DEWANJEE[3] and S. BHATTACHARYA[1]

Medicinal Plant Laboratory, Department of Botany, Bose Institute, 93/1 A.P.C. Road, Kolkata-700009, India[1]
Department of Bioscience and Biotechnology, Banasthali Vidypith, Rajasthan-304022, India[2]
Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032, India[3]

Abstract

Zephyr lily (*Zephyranthes grandiflora*), an important ornamental plant has been micropropagated *in vitro* after controlling microbial contamination by a pretreatment with 0.2 % *Bavistin* and 0.1 % *Pantomycin* for 4 h before final sterilization with 0.1 % mercuric chloride. In 67 % of the sterile cultures, 11 shoots on average were regenerated directly from basal half of bulb scales in Murashige and Skoog (MS) medium containing 3 % sucrose and 2 mg dm^{-3} benzylaminopurine (BAP). Shoots emerged in bunches on a basal achlorophyllous bulbous part. Combination of 2 mg dm^{-3} BAP with 1 mg dm^{-3} gibberellic acid (GA_3) enhanced shoot growth. Stout roots (maximum of 5 - 6 per shoot) were developed in presence of 1 mg dm^{-3} indole-3-butyric acid (IBA). Micro-bulbs showed potential of regeneration and could be used as secondary explants. The morphologically identical plants derived by *in vitro* propagation were genetically identical as shown by PCR based ISSR marker analysis of genomic DNA.

Additional key words: genetic stability, growth regulators, ISSR markers, micropropagation, zephyr lily.

Zephyranthes grandiflora Lindley (family *Amaryllidaceae*) commonly known as zephyr lily, is an ornamental plant native to Mexico. Large bright pink flowers of this species hold an important position in the cut flower market. Apart from the horticulture value the plant also shows medicinal properties. Recently transgenic tobacco expressing a *Z. grandiflora* agglutinin has shown increased resistance to aphids (Ye *et al*. 2009). Two types of *Z. grandiflora* plants - the sterile and the fertile are found in nature. The former do not yield seeds and are used by breeders to raise hybrids (Ogden 2007). Conventional propagation of *Zephyranthes* species and its hybrids by bulb division is slow, seasonal and inconsistent (Smith *et al.* 1999). Micropropagation provides an alternate means of large scale propagation but might induce somaclonal variation, making it mandatory to check genetic integrity. Keeping this in view, the present study was aimed to standardize a micro-propagation protocol for the production of genetically stable *Z. grandiflora* plants.

Bulbs of *Zephyranthes grandiflora* were collected from a wild spot at Kalimpong (West Bengal, India), and grown in the greenhouse of Bose Institute for several years. Freshly collected bulbs were taken, washed thoroughly under running tap water and the outer most scale removed. The bulbs were then immersed in *Teepol* (3 - 5 %, v/v) for 2 - 5 min with continuous shaking and rinsed with sterile distilled water. The clean bulbs were surface sterilized with 0.1 % $HgCl_2$ for 30 s, washed 3 - 4 times with sterile distilled water, cut into desired segments and placed on Murashige and Skoog (1962; MS) media supplemented with 3 % sucrose and 1 mg dm^{-3} benzylaminopurine (BAP). However, the culture showed microbial contamination and had to be discarded. Preventing contamination at the initial stages is the major challenge in culture of bulb tissues and requires application of various disinfectants (Liu and Burger 1986, Smith *et al.* 1999). As such, a revised sterilization protocol was devised following Ray and Bhattacharya (2008). Briefly, explants were treated with 0.2 % *Bavistin*

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Abbreviation: 2,4-D - 2,4-dichlorophenoxyacetic acid; BAP - 6-benzylaminopurine; dNTPs - deoxyribonucleotide triphosphate; EtBr - ethidium bromide; GA_3 - gibberellic acid-3; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; ISSR - inter simple sequence repeat; MS - Murashige and Skoog; NAA - 1-naphthalene acetic acid; PCR - polymerase chain reaction; TBE - tris borate EDTA.
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* Corresponding author; fax: (+91) 33 2350-0595, e mail: moumita_gangoadhyay@yahoo.co.uk

(*Carbendazim*, *BASF*, Mumbai, India) and 0.1 % *Pantomycin* (streptomycin sulfate and tetracyclin tetrachloride, *Aries*, Mumbai, India) for 2, 3, 4 and 5 h under continuous shaking on a magnetic stirrer, before final sterilization with 0.1 % $HgCl_2$ for 30 s. It was observed that 4 h pretreatment resulted in highest frequency (67 %) of non-contaminated living cultures after 30 d in MS medium containing 3 % sucrose and 1 mg dm^{-3} BAP.

Viable explants were identified by any of the signs such as swelling, greening or regenerating organs. Two types of explants were used in the experiment. For the first type, segmented bulbs (SB), made by two vertical sections (perpendicular to each other) of lower halves of the bulbs, giving four more or less equal pieces per bulb were taken. The second type constituted bulb scales (BS) obtained by peelings (outer and inner 2 - 3 layers) of the lower halves of bulbs with a small amount of attached stem tissue.

MS medium at pH 5.6, with 3 % sucrose, gelled with 0.8 % agar and autoclaved at 121 °C, 103 kPa for 20 min was used for *in vitro* culture. Explants (2 - 3 per 250 cm^3 culture flask, each containing 30 cm^3 medium) were placed vertically (SB, stem disc placing downwards) or horizontally (BS, dorsal or ventral side up) on medium augmented with either 1-naphthalene acetic acid (NAA) or 2,4-dichlorophenoxy acetic acid (2,4-D) at concentrations 0.5, 1, 1.5 and 2 mg dm^{-3} or BAP (1 mg dm^{-3}) singly and in combination with NAA (1 mg dm^{-3}). For further growth, explants showing organogenesis were transferred to medium containing BAP (1, 1.5, 2 and 2.5 mg dm^{-3}) singly or in combination with NAA (0.5 and 1 mg dm^{-3}). Prior to rooting, small shoots with prominent basal white tissue were cultured in MS medium supplemented with gibberellic acid (GA_3; 1 mg dm^{-3}) and BAP (2 mg dm^{-3}). Semisolid MS medium containing 2 % sucrose and either indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) (0.5, 1, and 1.5 mg dm^{-3}), gelled with 0.7 % agar was used for inducing roots. Cultures were maintained at 22 ± 2 °C and 18-h photoperiod (irradiance of 40 - 80 μmol m^{-2} s^{-1} provided by cool-white fluorescent tubes, *Philips*, Gurgaon, India). Each set of experiment consisted of 10 replicas and each experiment was repeated at least thrice. Data were recorded from cultures showing more or less similar results, discarding the extreme odds if any. Basal achlorophyllous micro-bulb like part of *in vitro* raised shoots were incised longitudinally (half the way along the longitudinal axis but still attached at the tip) and cultured as secondary explants on fresh MS medium containing BAP (2 mg dm^{-3}) singly or BAP (1 mg dm^{-3}) and NAA (0.5 mg dm^{-3}) in combination.

Well-rooted plantlets, after complete removal of agar from roots were transferred to small plastic pots containing *Soilrite*-soil mixture (1:1) and kept under tissue culture conditions till the plantlets resumed growth. The plants were then transferred to glasshouse, transplanted to earthen pots and maintained for 6 months and finally planted in the medicinal plant garden of Bose Institute.

Inter simple sequence repeat (ISSR) marker was used (self designed and purchased from *Bangalore Genie*, Bangalore, India) for evaluation of clonal fidelity among the acclimatized micropropagated plants. Genomic DNA was isolated from randomly selected bulb tissues of *in vitro* regenerated acclimatized plants using the procedure of mini preparation of plant DNA by Dellaporta *et. al.* (1993). The PCR reaction was carried out in 25 mm^3 volume with 20 ng DNA, 100 ng of primer, 3.5 mm^3 2.5 mM dNTPs; 0.5 U Taq DNA polymerase, 2.5 mm^3 10× buffer and 15.5 mm^3 water. PCR conditions were 94 °C for 5 min; followed by 35 cycles 94 °C for 45 s, 45 °C for 45 s and 72 °C for 90 s and final extension for 7 min at 72 °C followed by 5 min at 4 °C. The PCR product was analyzed along with a 100 bp DNA ladder (purchased from *Bangalore Genie*) on a 1.5 % agarose gel stained with EtBr and 1× TBE as running buffer at 65 - 70 V. The gels were checked under UV radiation and photographed in gel documentation system (*Gel-doc 1000*, *Bio-Rad*, Hercules, USA).

A randomized block design was used to set up all the experiments. Data were examined by analysis of variance (*ANOVA*) significantly ($P < 0.05$) different means were compared using Duncan multiple range test using *SPSS* software (*17.0.0*, *SPSS Inc.*, Chicago, USA).

In vitro morphological changes of *Z. grandiflora* bulb explants in cultures containing various growth regulators in MS were observed for one month. The explants, irrespective of type of growth regulator added, started swelling within one week of culture (Fig. 1*A*). Subsequent to swelling, greening occurred in presence of all tested growth regulators except 2,4-D. After fourth week, explants in BAP singly or BAP and NAA in combination produced small, white, bead like structures that turned gradually green at their tips and behaved like shoot primordium. However, 2,4-D and NAA (singly) did not induce organogenesis.

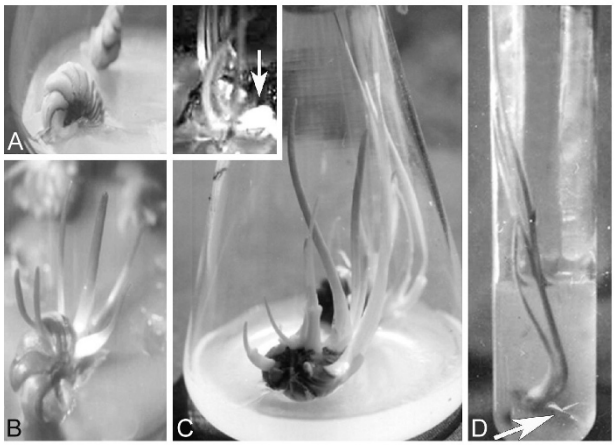

Fig. 1. Different developmental stages of micropropagation of *Z. grandiflora*: swelled segmented bulb explant (*A*), shoot regeneration from segmented bulb (*B*), bunch of shoots in bulb scale culture (*C*), older culture showing secondarily developed shoots on decaying explant (*C-inset*), emerging root from shoot base (*D*).

Table 1. Effect of BAP singly and in combination with NAA [mg dm^{-3}] on *Z. grandiflora* shoot regeneration from bulb scales (BS) and segmented bulbs (SB). Data were recorded at the end of second subculture (60 d). Results are mean ± SD of 10 independent experiments (n = 30) and means with the same letters are not significantly different at $P \leq 0.05$.

NAA	BAP	Responding explants [%]		Number of shoots [explant^{-1}]	
		BS	SB	BS	SB
0	1.0	60^{bc}	48^{a}	5.39 ± 2.60^{fg}	4.10 ± 1.60^{d}
	2.0	68^{c}	54^{e}	11.39 ± 2.66^{h}	7.10 ± 2.00^{f}
	2.5	59^{b}	46^{a}	6.61 ± 4.43^{g}	4.70 ± 3.10^{e}
0.5	1.0	52^{ab}	36^{b}	3.39 ± 2.06^{de}	2.37 ± 1.82^{c}
	2.0	62^{b}	41^{c}	6.50 ± 2.36^{g}	4.20 ± 2.04^{d}
	2.5	59^{a}	39^{b}	5.22 ± 3.00^{fg}	3.70 ± 4.03^{d}
1.0	1.0	53^{a}	37^{b}	3.17 ± 2.28^{cde}	1.88 ± 1.98^{bc}
	2.0	48^{a}	29^{d}	2.06 ± 1.98^{bcd}	1.01 ± 2.40^{ab}
	2.5	40^{a}	27^{d}	1.06 ± 1.16^{ab}	0.77 ± 1.99^{a}

To optimize organogenesis, only those cultures, showing shoot primordial structures were transferred to media containing 1, 2 and 2.5 mg dm^{-3} BAP singly or in combination with NAA (0.5 and 1 mg dm^{-3}). BAP at 2 mg dm^{-3} concentration was found to be most effective for shoot regeneration whereby at an average, 11 and 7 shoots were produced in BS and SB explants, respectively (Table 1). BAP (2 mg dm^{-3}) and NAA (0.5 mg dm^{-3}) in combination was almost equally effective in this respect but regeneration induced by this combination caused callusing. Shoots in SB were initiated from the basal tissue plate and appeared later on the surface of the swelled scales (Fig. 1*B*). In BS explants, shoots were observed to initiate from the tip of white achlorophyllous organs developed at the basal end of the explant (Fig. 1*C*). With time, bunch of new shoots

Fig. 2. Micropropagated *Z. grandiflora* plant transferred to plastic pot kept under culture conditions (*A*), plant in earthen pot in greenhouse (*B*), 6-month-old micropropagated flowering *Z. grandiflora* plants in greenhouse (*C*).

Table 2. *In vitro* rooting of *Z. grandiflora* microshoots treated with IBA and IAA at different concentrations after 30 d culture. Means ± SD, n = 30, means with the same letters are not significantly different at $P \leq 0.05$.

Growth regulator	Concentration [mg dm^{-3}]	Rooting [%]	Root number [shoot^{-1}]
IBA	0.5	59.26^{c}	3.89 ± 0.78^{c}
	1.0	85.19^{d}	5.33 ± 1.58^{d}
	1.5	33.33^{ab}	2.33 ± 1.22^{b}
IAA	0.5	22.22^{a}	2.56 ± 1.33^{b}
	1.0	48.15^{bc}	5.67 ± 1.32^{d}
	1.5	18.51^{a}	0.78 ± 0.67^{a}

were found to develop from the base of older ones (Fig. 1*C* - inset). The excised basal part of shoots when used as secondary explant also produced large number of leafy shoots. A similar organogenesis potential of the basal achlorophyllous tissue from shoot clumps of *Narcissus* has been reported previously by Chow *et al.* (1993). In addition to BAP (2 mg dm^{-3}), GA_3 (1 mg dm^{-3}) was essential for growth of *Z. grandiflora* microshoots. Subsequently, basal tissue enlarged, took perfect shape of micro-bulbs and turned green.

IBA (1 mg dm^{-3}), was found to be suitable in inducing rooting (5.67 numbers per shoot, Table 2). Roots were stout but few in number and without any laterals at the initial stage (Fig. 1*D*). Plantlets with well developed roots were transferred to plastic pots (Fig. 2*A*) with *Soilrite*-soil mix and covered with plastic sheet to avoid desiccation and kept under tissue culture conditions at least two week from there they were transferred to greenhouse where the plants were hardened with about 70 % survival rate (Fig. 2*B*). Healthy plants were transferred to the medicinal plant garden of Bose Institute. Some plants were also maintained in the greenhouse (Fig. 2*C*).

Explant and growth regulators play an important role in micropropagation. Twin scales (an explant with two scale bases attached to the bulbs at the basal plate) are widely used in micropropagating lilies (Huang *et al.* 1990, Kumar 2005). Organogenesis from explants like small sections of bulb scales (Han *et al.* 2004), shoot tips (Shivakumar and Krishnamurthy 2004), cormlets and whole corm (Mukhopadhyay *et al.* 2002, Sharma *et al.* 2008) have also been reported. In the present study, half scales (from the basal half of the bulb) and segmented bulbs with attached basal plate were found to be efficient sources for regeneration of *Z. grandiflora* shoots. Action of growth regulators, BAP and NAA in particular, in shoot induction has been reported by several workers on micropropagation of bulbous lilies (Mukhopadhyay *et al.* 2002, Azadi and Khosh-Khui 2007, Sharma *et. al.* 2008).

To access the genetic stability within micropropagated plants, various DNA marker techniques are used (Saker *et al.* 2006, Feyissa *et al*. 2007, Xing *et al*. 2010). ISSR, one of the PCR based reliable molecular marker techniques has largely been employed in recent years for

checking genetic uniformity of a number of micropropagated plant species as it is a relatively simple, low cost, non radioactive technique requiring less amount of DNA and no prior primer sequence information (Joshi and Dhawan 2007, Huang *et al.* 2009). In the present study, out of twenty ISSR primers used, eight (Table 3) showed clearly visible bands. The ISSR profile of micropropagated *Z. grandiflora* plants revealed that all the DNA samples produced monomorphic bands (Fig. 3). The monomorphic banding pattern indicates genetic uniformity in the tissue culture-derived plants. The direct mode of regeneration of *Z. grandiflora* bypassing callus phase might be responsible in maintaining genetic stability of the clonal population.

Table 3. List of ISSR primers showing positive PCR amplification in micropropagated *Z. grandiflora* plants.

Primer	Primer Sequence (5′ - 3′)	Annealing temp. [° C]
IS-61	GAGAGAGAGAGAGAGAT	45
IS-62	GATAGATAGATAGATAGATA	36
IS-63	AGAGAGAGAGAGAGAGC	45
IS-64	TTTATTATTATTATT	30
IS-65	AGAGAGAGAGAGAGAGT	45
IS-8	AGAGAGAGAGAGAGAGC	52
IS-9	TGTGTGTGTGTGTGTA	46
IS-11	CACACACACACACACAG	52

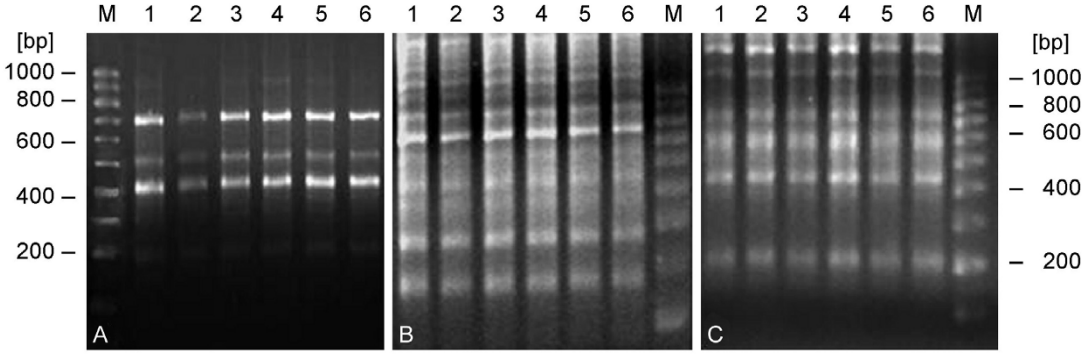

Fig. 3. Portions of three typical agarose gels showing ISSR profiles generated with primers IS8 (A), IS9 (B) and IS11 (C). *Lane* M: 100 bp marker, *lanes* 1 - 6: DNA extracted from randomly selected bulb of acclimatized plants.

The micropropagation protocol reported herein will be useful for mass multiplication of genetically stable *Z. grandiflora* from limited donor plants within a short time. The protocol was optimized by manipulations of different plant growth regulators, for enhanced multiplication. From an established culture, plantlets can be produced regularly if *in vitro* grown bulbs are used as secondary explants. In conclusion it can be said that this protocol provides a starting point for the *ex situ* conservation of this ornamental as well as medicinally important plant.

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