BRIEF COMMUNICATION

Efficient and repetitive production of leaf-derived somatic embryos of *Oncidium*

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Abstract

Oncidium cultivars gave different embryogenic responses of leaf explants when affected by auxins (2,4-D, IAA, IBA and NAA), cytokinins (2iP, BA, kinetin, TDZ and zeatin), sucrose, NaH_2PO_4, casein hydrolysate, peptone, and glutamine. The best embryogenic responses of cv. Sweet Sugar were at 20 g dm^{-3} sucrose, 85 mg dm^{-3} NaH_2PO_4 and 3 mg dm^{-3} kinetin, respectively. The development of somatic embryos on leaf explants of cv. Sweet Sugar was delayed for about 10 - 20 d in comparison with cv. Gower Ramsey. On growth regulator-free medium, about 40 % of leaf derived embryos of cv. Gower Ramsey were fused together in their basal parts and so called multiple-state embryos. However, under the same condition, the embryos of cv. Sweet Sugar were all in multiple-state form.

Additional key words: direct somatic embryogenesis, genotype, medium composition.

In *Oncidium*, plant regeneration through somatic embryogenesis has been established in cv. Gower Ramsey (Chen *et al.* 1999, Chen and Chang 2000a). The systems were further used to investigate the effects of exogenous auxins and cytokinins (Chen and Chang 2001, Wu *et al.* 2004), tissue culture conditions, explant characteristics (Chen and Chang 2002), ACC, ethylene inhibitors (Chen and Chang 2003a), GA_3 and growth retardants (Chen and Chang 2003b) on somatic embryogenesis. In another economical important *Oncidium* cultivar Sweet Sugar, a quit different response was found on *in vitro* morphogenesis from flower stalk explants as compare with cv. Gower Ramsey (Chen and Chang 2000b). Objective of this report was induction of the embryogenic culture from leaf explants of cv. Sweet Sugar and determination of differences in direct embryo formation as compare with cv. Gower Ramsey.

Two-month-old *in vitro*-grown donor plantlets of *Oncidium* cv. Sweet Sugar were obtained from flower stalk explants (Chen and Chang 2000b) and were maintained on growth regulator-free half-strength Murashige and Skoog (1962) medium supplemented with [mg dm^{-3}]: myo-inositol (100), niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (2.0), peptone (1000), NaH_2PO_4 (170), sucrose (20000), and *Gelrite*™ (2200). Plant growth regulators were added prior to autoclaving. The pH of the media was adjusted to 5.2 with 1 M KOH or HCl prior to autoclaving for 15 min at 121 ºC. Explants were incubated in 20 × 150 mm culture tubes under irradiance of 28 - 36 μmol m^{-2} s^{-1} (daylight fluorescent tubes *FL-30D/29*, 40 W, *China Electric Co.*, Taipei, Taiwan), 16-h photoperiod and temperature of 26 ± 1 ºC.

For induction of direct embryo formation from leaf explants, tips (about 1 cm in length) were taken from young leaves of *in vitro*-grown plantlets and were placed adaxial-side-up on the surface of culture medium without any plant growth regulators. Further, pieces of leaf-

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; 2iP - N^6-[2-isopentenyl]-adenine; BA - N^6-benzyladenine; kinetin - 6-furfurylaminopurine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog; NAA - naphthaleneacetic acid; TDZ - 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (thidiazuron); zeatin - 6-[4-hydroxy-3-methylbut-2-enylamino]purine.

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derived embryogenic masses (1 mm^3) were placed on the surface of culture medium to test the effects of 0, 0.3, 0.6 and 0.9 mg dm^{-3} TDZ on embryo formation and spontaneous plantlet formation.

Effect of medium composition was tested on leaf tip segments (about 1 cm) placed adaxial side up on the surfaces of media. Sucrose (0, 10, 20, 30 and 60 g dm^{-3}), Na_2HPO_4 (0, 17, 42.5, 85 and 170 mg dm^{-3}), casein hydrolysate, peptone and glutamine (*Sigma*, St. Louis, USA) (0, 0.1, 0.5 and 1 g dm^{-3}) were added to the media. The frequency of embryogenesis and average numbers of embryos per explant were scored on four leaf regions (leaf tip, adaxial, abaxial side and cut end).

Leaf tip segments (about 1 cm long) were also used as explants to test four auxins (2,4-D, IAA, IBA and NAA) and five cytokinins (2iP, BA, kinetin, TDZ and zeatin) at concentrations of 0.3, 1 and 3 mg dm^{-3} on direct somatic embryogenesis. The frequency of embryogenesis and average numbers of embryos per explant were scored on four leaf regions (leaf tip, adaxial, abaxial side and cut end).

Cultures were examined and photographed with a stereozoom microscope (*SZH*, *Olympus*, Tokyo, Japan). Samples for scanning electron microscopy were fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 4 h at 4 °C, dehydrated in ethanol (Dawns 1971), critical point dryer (*HCP-2*, *Hitachi*, Kyoto, Japan), and coated with gold in an ion coater (*IB-2*, *Giko Engineering Co.*, Japan). A scanning electron microscope (*DSM-950*, *Carl Zeiss*, Jena, Germany) was used for examination and photography of the specimen. Treatment means were compared using Duncan's multiple range test (Duncan 1955).

Somatic embryos directly formed from leaf explants of cv. Sweet Sugar after 20 - 30 d of culture on hormone-free 1/2 MS medium (Fig. 1*A*,*C*). After another 20 d in

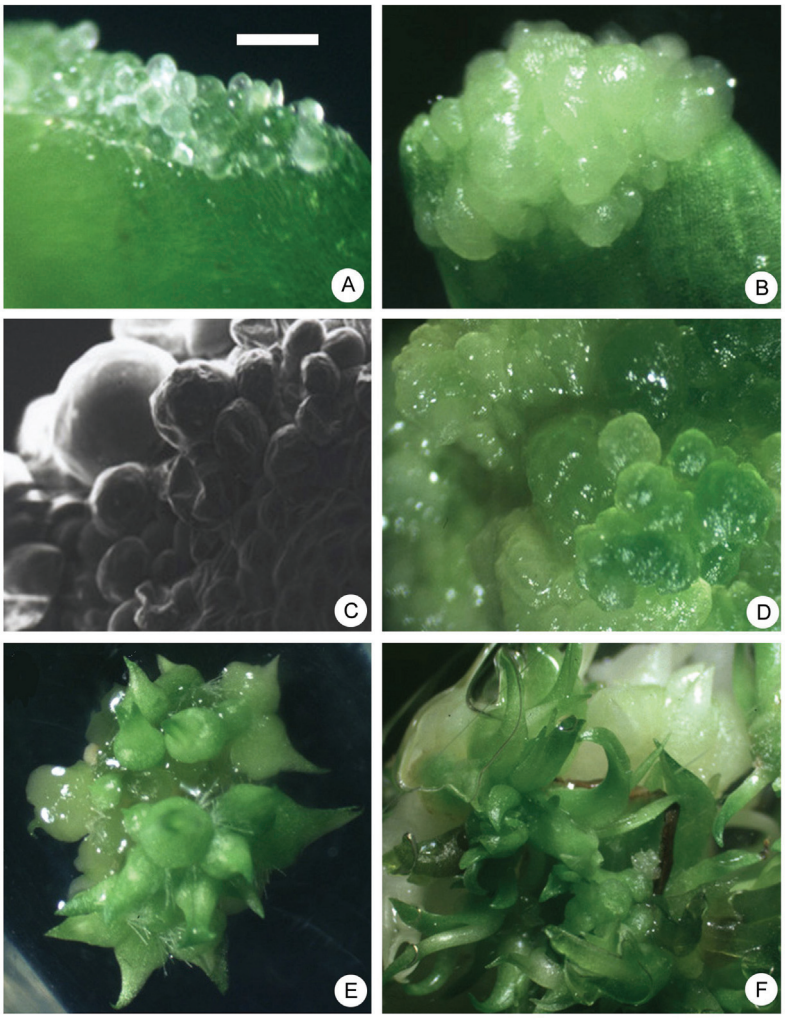

Fig. 1. Direct somatic embryogenesis from leaf explants of *Oncidium* cv. Sweet Sugar. *A* - somatic embryos formed from the leaf explant on a hormone-free medium (*bar* = 4.8 mm); *B* - somatic embryos enlarged and developed into young protocorms (*bar* = 1.8 mm); *C* - scanning electron microscopic photograph of leaf derived embryos after 20 d in culture (*bar* = 330 µm); *D* - the subculture leaf-derived embryogenic mass bearing embryos (*bar* = 2.1 mm); *E* - young protocorms derived from subculture embryogenic mass (*bar* = 3.2 mm); *F* - regenerated plantlets derived from embryos (*bar* = 3.1 mm).

Table 1. Effect of TDZ on proliferation and embryo formation of leaf-derived embryogenic mass of *Oncidium* cv. Sweet Sugar. Data were scored after 30 d in culture. Means of 7 replicates with the same letters are not significantly different at $P < 0.05$ (Duncan 1955).

TDZ [mg dm^{-3}]	Embryogenic mass [mg]	Mass of protocorms [mg]	Number of shoots
0	536 a	32 c	18.3 a
0.3	423 b	340 b	7.1 b
0.6	410 b	349 ab	5.1 b
0.9	412 b	371 a	4.9 b

Table 2. Effect of sucrose on direct somatic embryogenesis on leaf explants of *Oncidium* cv. Sweet Sugar. Data were scored after 40 d in culture. Means of 7 replicates with the same letters are not significantly different at $P < 0.05$.

Sucrose [g dm^{-3}]	Embryogenesis [%] tip	adaxial side	abaxial side	cut end	Embryo number [explant^{-1}]
0	0 b	0 b	0 a	0 b	0 b
10	25.0 a	21.4 a	0 a	28.6 a	11.0 a
20	25.0 a	10.7 ab	0 a	25.0 a	7.1 a
30	17.9 ab	14.3 ab	0 a	25.0 a	7.3 a
60	21.4 ab	7.1 ab	0 a	21.4 a	6.4 a

Table 3. Effect of NaH_2PO_4 on direct somatic embryogenesis on leaf explants of *Oncidium* cv. Sweet Sugar. Data were scored after 40 d in culture. Means of 5 replicates with the same letters are not significantly different at $P < 0.05$.

NaH_2PO_4 [mg dm^{-3}]	Embryogenesis [%] tip	adaxial side	abaxial side	cut end	Embryo number [explant^{-1}]
0	15 a	25 a	0 a	15 b	4.2 a
17.0	30 a	30 a	0 a	10 b	8.0 a
42.5	40 a	25 a	0 a	25 ab	9.2 a
85.0	50 a	40 a	0 a	45 a	13.6 a
170.0	40 a	35 a	0 a	30 ab	11.4 a

culture, embryos enlarged and young protocorms were obtained (Fig. 1*B*). Under the same conditions, it only took about one month to obtained embryo-derived protocorms from leaf explants of cv. Gower Ramsey (Chen and Chang 2001). In cv. Sweet Sugar, 100 % of the leaf-derived embryos fused together in their basal regions and showed multiple-state form on hormone-free medium (Fig. 1*B*). However, only about 40 % of leaf explants of cv. Gower Ramsey formed multiple-state embryos in the same condition. The multiple-state embryos of cv. Sweet Sugar formed shoots after one month on the same medium. Normal regenerated plantlets with 3 - 4 leaves and 2 - 3 roots could be obtained from these multiple-state embryos after another one month on the same hormone-free medium.

The small pieces (1 × 1 mm^2) from embryo clusters could be subculture on hormone-free medium, and had highly competence to from secondary embryos (Fig. 1*D*). However, when most of the embryos already developed into protocorms (Fig. 1*E*), the leaf-derived tissues had been unsuitable for continuous embryo production. These secondary embryos converted into plantlets after 40 - 50 d of culture on hormone-free medium (Fig. 1*F*). TDZ at 0.3 - 0.9 mg dm^{-3} significantly enhanced fresh mass of embryos, but reduced number of shoots.

Table 4. Effects of casein hydrolysate, peptone and glutamine on direct somatic embryogenesis on leaf explants of *Oncidium* cv. Sweet Sugar. Data were scored after 40 d in culture. Means of 5 replicates with the same letters are not significantly different at $P < 0.05$.

Compound [mg dm^{-3}]		Embryogenesis [%] tip	adaxial side	abaxial side	cut end	Embryo number [explant^{-1}]
Control		15 ab	15 a	0 a	10 ab	3.4 a
Casein	0.1	0 b	10 a	0 a	5 ab	2.2 a
hydrolysate	0.5	20 ab	20 a	0 a	15 ab	5.8 a
	1.0	20 ab	10 a	0 a	0 b	3.4 a
Peptone	0.1	10 ab	5 a	0 a	0 b	1.4 a
	0.5	5 ab	5 a	0 a	10 ab	2.0 a
	1.0	0 b	10 a	0 a	5 ab	1.6 a
Glutamine	0.1	5 ab	10 a	0 a	0 b	1.4 a
	0.5	15 ab	30 a	0 a	20 a	7.0 a
	1.0	30 a	10 a	0 a	5 ab	7.4 a

Table 5. Effects of five cytokinins on direct somatic embryogenesis on leaf explants of *Oncidium* cv. Sweet Sugar. Data were scored after 40 d in culture. Means of 7 replicates with the same letters are not significantly different at $P < 0.05$.

Cytokinins [mg dm^{-3}]		Embryogenesis [%] tip	adaxial side	abaxial side	cut end	Embryo number [explant^{-1}]
Control		3.6 b	0 c	0 b	1.8 c	0.6 c
2iP	0.3	7.1 b	3.6 bc	0 b	3.6 c	1.4 bc
	1.0	7.1 b	7.1 bc	0 b	10.7 bc	2.4 bc
	3.0	17.9 ab	14.3 bc	0 b	21.4 abc	5.7 bc
BA	0.3	7.1 b	3.6 bc	0 b	3.6 c	1.6 bc
	1.0	7.1 b	7.1 bc	0 b	7.1 bc	2.9 bc
	3.0	14.3 ab	14.3 abc	0 b	10.7 bc	5.0 bc
Kinetin	0.3	32.1 a	17.9 abc	0 b	17.9 abc	7.1 b
	1.0	17.9 ab	21.4 ab	0 b	25.0 ab	7.0 b
	3.0	32.1 a	28.6 a	0 b	35.7 a	14.3 a
TDZ	0.3	10.7 b	3.6 bc	0 b	3.6 c	4.1 bc
	1.0	14.3 ab	10.7 bc	0 b	10.7 bc	4.9 bc
	3.0	28.6 a	10.7 bc	0 b	14.3 bc	12.1 a
Zeatin	0.3	7.1 b	3.6 bc	0 b	14.3 bc	3.4 bc
	1.0	14.3 ab	10.7 bc	0 b	17.9 abc	4.4 bc
	3.0	21.4 ab	21.4 ab	3.6 a	10.7 bc	7.1 b

Sucrose at 20 g dm^{-3} was the most suitable concentration for direct somatic embryogenesis from leaf explants of cv. Sweet Sugar (Table 2). In NaH_2PO_4 treatment, 85 mg dm^{-3} had a significant higher embryogenic response on cut end when compared with the control treatment (Table 3). Three organic forms of nitrogen, casein hydrolysate, peptone, and glutamine had no significant effects on direct embryogenesis (Table 4). In cv. Gower Ramsey, the same concentrations of sucrose, NaH_2PO_4, casein hydrolysate, peptone, and glutamine were tested on direct embryogenesis, and the best responses were at 10 - 20 g dm^{-3} sucrose, 170 mg dm^{-3} NaH_2PO_4 and 0.5 g dm^{-3} peptone (Chen and Chang 2002).

In cv. Gower Ramsey, all exogenous auxins tested (2,4-D, IAA, IBA and NAA) significantly retarded direct embryogenesis (Chen and Chang 2001). However, the same concentrations of auxins had no significant effects on direct embryo formation in cv. Sweet Sugar (data not shown). In cv. Gower Ramsey, the highest frequencies of embryo formation between cytokinin treatments on leaf tips, adaxial sides and cut ends of explants were at 1 mg dm^{-3} TDZ, 1 mg dm^{-3} 2iP and 0.3 mg dm^{-3} kinetin, respectively (Chen and Chang 2001). In addition, 1 mg dm^{-3} TDZ gave the highest number of embryos per explant in cv. Gower Ramsey (Chen and Chang 2001). However, in cv. Sweet Sugar, the best responses were all obtained at 3 mg dm^{-3} kinetin (Table 5).

Oncidium cv. Sweet Sugar had different requirements in cytokinin and medium compositions for induction of direct embryogenesis from leaf explants as compare with cv. Gower Ramsey. The best response on direct somatic embryogenesis of cv. Sweet Sugar was obtained on the modified 1/2 MS medium supplemented with 20 g dm^{-3} sucrose, 85 mg dm^{-3} NaH_2PO_4 and 3 mg dm^{-3} kinetin.

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