BIOLOGIA PLANTARUM 46 (3): 455-458, 2003

BRIEF COMMUNICATION

1-aminocyclopropane-1-carboxylic acid enhanced direct somatic embryogenesis from *Oncidium* leaf cultures

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Abstract

Influence of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and two ethylene inhibitors, silver nitrate ($AgNO_3$) and cobalt chloride ($CoCl_2$), on direct somatic embryogenesis were tested *in vitro* using leaf cultures of *Oncidium* cv. Gower Ramsey. Leaf cells of tips, adaxial sides and cut ends could directly form somatic embryos on a hormone-free 1/2-strength MS medium. The frequency of embryo-producing explants was 55, 52.5 and 30 %, respectively. The embryo numbers per embryo-producing explant was 20.3. ACC at lower concentrations (5 and 10 μM) significantly retarded direct embryo formation from cut ends. However, higher concentrations of ACC (20 and 50 μM) significantly promoted embryogenic response of leaf tips and adaxial sides. All concentrations of $AgNO_3$ and $CoCl_2$ significantly retarded direct embryo formation. The best response was found on 20 μM of ACC, and the frequency of embryo-producing explants were 90, 85 and 35 % on leaf tips, adaxial sides and cut ends, respectively. The embryo numbers per embryo-producing explant was 32.2.

Additional key words: ACC, cobalt chloride, ethylene inhibitor, ethylene precursor, silver nitrate.

Direct somatic embryogenesis has often been considered to be a suitable method for mass propagation and for the regeneration of transgenic plants. In addition, direct somatic embryogenesis also provides a model system for studying the morphology and the effect of internal and environmental factors on non-zygotic embryos (Conger *et al.* 1983). In *Oncidium* orchids, embryogenic callus cultures were induced *in vitro* by using root, stem and leaf (Chen and Chang 2000a) and flower stalk internode (Chen and Chang 2000b) as explants, and healthy plantlets were successfully obtained from the callus cultures. Furthermore, direct somatic embryogenesis from leaf explants was established on a modified 1/2-strength Murashige and Skoog (MS) medium (Chen *et al.* 1999). We have used the leaf culture system to investigate the influence of exogenous auxins, exogenous cytokinins (Chen and Chang 2001), tissue culture conditions, explant characteristics (Chen and Chang 2002) on direct somatic embryogenesis. Here we describe the effects of ethylene precursor 1-aminocyclo-propane-1-carboxylic acid (ACC) and two ethylene inhibitors, silver nitrate ($AgNO_3$) and cobalt chloride ($CoCl_2$) on direct somatic embryogenesis from *Oncidium* leaf explants.

Two-month-old *in vitro*-grown donor plantlets of *Oncidium* sp. cv. Gower Ramsey were obtained from flower-stalk bud-derived protocorm-like-bodies (Chen *et al.* 1999). Plant growth regulators were sterilized by filtration using 0.2 μm filters (*Gelman Sciences*, USA), and added to a 1/2-strength Murashige and Skoog (1962; MS) basal medium. Explants were incubated in Petri dishes (*Alpha Plus Scientific Corp.*, Taiwan, China) under a 16-h photoperiod at irradiance of 28 - 36 μmol m^{-2} s^{-1} (daylight fluorescent tubes *FL-30D/29*, 40 W, *China Electric Co.*, Taipei, Taiwan) and temperature of 26 ± 1 °C. Three independent experiments were performed to evaluate the effects of ethylene precursor (ACC), two ethylene inhibitors, $AgNO_3$ and $CoCl_2$, and (1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ, a synthetic cytokinin)

Received 7 September 2001, *accepted* 25 April 2002.
Abbreviations: ACC - 1-aminocyclopropane-1-carboxylic acid; MS - Murashige and Skoog; TDZ - thidiazuron.
Acknowledgments: This study was support by Academia Sinica, and National Science Council, Republic of China. Experiments were conducted at the Institute of Botany, Academia Sinica at Taipei, Taiwan, Republic of China.
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on direct embryo formation from leaf explants of *Oncidium*. The percentage of embryo-producing explants and the embryo numbers per embryo-producing explant were determined for each trial. ACC, $AgNO_3$ and $CoCl_2$ (0.5, 2.5, 5, 10, 20 μM) were used in experiment 1 (see Table 1); ACC (5, 10, 20 μM), $AgNO_3$ (0.5, 2.5, 5 μM) and $CoCl_2$ (0.5, 2.5, 5 μM) were used in experiment 2 (see Table 2); TDZ (1 μM), ACC (20 μM), 1 μM TDZ + 20 μM ACC were used in experiment 3 (see Table 3). Forty replicates were taken for each treatment, and eight explants were planted in each dish. Observations were made after 40 - 60, 8 and 3 - 9 weeks of growth in experiment 1, 2 and 3, respectively. Differences between means were scored with Duncan's multiple range test (Duncan 1955). Cultures were examined with a stereozoom microscope (*SZH*, *Olympus*).

On a hormone-free medium, 40 % of cultured leaf explants formed 6.4 somatic embryos after 40 d in culture, and the frequency of embryo-producing explants was raised to 62.6 % and the embryo number per embryo-producing explant was 22.2 after 60 d in culture (Table 1). At lower concentrations (0.5 - 5 μM) of ACC the embryogenic response was slightly retarded either after 40 or 60 d in culture (Table 1). However, ACC at 20 μM significantly promoted direct embryo formation from leaf explants, and there were 90 % of explants formed from 34.6 embryos after 60 d in culture (Table 1).

Table 1. Effects of ACC, $AgNO_3$ and $CoCl_2$ on direct somatic embryogenesis from 1 cm long leaf explants of *Oncidium*. The frequency of embryo-producing explants and average embryo numbers per embryo-producing explants were scored after 40 and 60 d in culture. Means of 40 replicates. These with the same letters are not significantly different at $P < 0.05$ (Duncan 1955).

Treatment	Conc. [μM]	Embryogenesis [%] 40 d	60 d	Number of embryos [explant^{-1}] 40 d	60 d
Control		40.0 b	62.5 b	6.4	22.2
ACC	0.5	30.0 b	52.5 bc	5.8	20.2
	2.5	40.0 b	42.5 c	6.2	21.0
	5.0	37.5 b	47.5 bc	6.0	20.5
	10.0	42.5 b	60.0 b	8.2	24.8
	20.0	60.0 a	90.0 a	15.2	34.6
$AgNO_3$	0.5	5.0 c	15.0 d	2.0	6.7
	2.5	5.0 c	12.5 d	2.0	4.0
	5.0	0.0 c	12.5 d	0	7.0
	10.0	0.0 c	10.0 d	0	6.7
	20.0	0.0 c	5.0 d	0	5.0
$CoCl_2$	0.5	12.5 c	15.0 d	4.2	12.5
	2.5	5.0 c	15.0 d	2.5	12.3
	5.0	5.0 c	12.5 d	2.0	10.0
	10.0	0.0 c	5.0 d	0	6.5
	20.0	0.0 c	2.5 d	0	5.0

All the concentrations of $AgNO_3$ tested significantly retarded direct embryo formation from leaf explants (Table 1). The severe case was found on the concentration of 20 μM, there was only 5 % of explants formed 5 embryos after 60 d in culture (Table 1). Likewise, $CoCl_2$ retarded direct embryo formation from leaf explants at all concentration tested (Table 1).

Table 2. Effects of ACC, $AgNO_3$ and $CoCl_2$ on direct somatic embryogenesis from various regions of *Oncidium* leaf explants. The frequency of embryo-producing explants and average embryo numbers per embryo-producing explants were scored after 8 weeks in culture. Means of 40 replicates. These with the same letters are not significantly different at $P < 0.05$ (Duncan 1955). No embryo was found on abaxial side.

Treatment	Conc. [μM]	Embryogenesis [%] leaf tip	adax. side	cut end	Embryos [explant^{-1}]
Control		55.0 b	52.5 b	30.0 a	20.3
ACC	5	37.5 b	40.0 b	12.5 b	20.4
	10	47.5 b	50.0 b	15.0 b	20.1
	20	90.0 a	85.0 a	35.0 a	32.2
	50	85.0 a	85.0 a	35.0 a	30.5
$AgNO_3$	0.1	17.5 c	15.0 c	2.5 b	8.5
	0.5	15.0 c	15.0 c	2.5 b	6.3
	2.5	10.0 c	0.0 c	2.5 b	3.3
	5.0	0.0 c	0.0 c	12.5 b	6.0
$CoCl_2$	0.1	15.0 c	15.0 c	10.0 b	11.8
	0.5	15.0 c	12.5 c	10.0 b	11.5
	2.5	12.5 c	12.5 c	10.0 b	11.2
	5.0	12.5 c	12.5 c	5.0 b	8.0

In experiment 2, we test the influence of ACC, $AgNO_3$ and $CoCl_2$ on direct somatic embryogensis from leaf tip, adaxial, abaxial side and cut end. The embryogenic responses of leaf tips, adaxial, abaxial sides and cut ends were 55.0, 52.5, 0 and 30.0 % after 8 weeks in culture, respectively. ACC at 5 and 10 μM resulted in no significant effect on direct embryo formation from leaf tips, adaxial and abaxial sides of leaf explants, but significantly retarded embryogenic response of cut ends. However, ACC at 20 and 50 μM significantly promoted embryogenic response of leaf tips and adaxial leaf regions, and these explants formed 32.2 and 30.5 embryos, respectively (Table 2). Low dosage of $AgNO_3$ (0.1 μM) significantly retarded embryogenic response of all leaf regions except abaxial leaf region, and the embryo numbers per embryo-producing explant was 8.5 (Table 2). In addition, higher concentrations of $AgNO_3$ (0.5, 2.5 and 5 μM) also significantly retarded direct embryo formation from leaf explants, and there were no embryogenic response on leaf tips and adaxial sides of explants at 5 μM $AgNO_3$ (Table 2). In the present of 0.1 - 5 μM $CoCl_2$, the embryogenic response were

significantly retarded on leaf tips, adaxial sides and cut ends, and one explant formed only 8 - 11.8 embryos (Table 2). We also found no embryos on abaxial leaf regions in all treatments (Table 2).

After 3 weeks in culture, only ACC (20 μM) plus TDZ (1 μM) significantly promoted direct embryo formation (Table 3). After 6 - 9 weeks, all three treatments gave significant higher embryogenic response in comparison with the control treatment. 20 μM ACC results in a highest embryo numbers of per embryo-producing explant after 9 weeks in culture.

Table 3. Effects of 20 μM ACC and 1 μM TDZ on direct embryogenesis from *Oncidium* leaf explants. The frequency of embryo-producing explants and average embryo numbers per embryo-producing explants were scored on each explants after 3, 6 and 9 weeks in culture. Means of 40 replicates. These with the same letters are not significantly different at $P < 0.05$ (Duncan 1955).

Treatment	Embryogenesis [%]			Embryos [explant^{-1}]		
	3 wk	6 wk	9 wk	3 wk	6 wk	9 wk
Control	25.0 b	40.0 b	57.5 b	2.0	6.3	21.8
ACC	32.5 ab	75.0 a	90.0 a	2.4	16.8	36.5
TDZ	40.0 ab	77.5 a	80.0 a	3.8	10.5	28.1
ACC+TDZ	45.0 a	75.0 a	90.0 a	4.2	11.2	30.2

The embryogenic cells of three regions of *Oncidum* leaf explants (leaf tips, adaxial sides and abaxial sides) could directly form somatic embryos on a hormone-free medium. By contrast, we found no embryos formed from abaxial leaf region. In a previous report, we found that exogenous cytokinins (2iP, BA, kinetin, TDZ and zeatin) could enhance direct embryo formation, but exogenous auxins (2,4-D, IAA, IBA and NAA) were inhibitory (Chen and Chang 2001). In this paper, we demonstrated that ethylene precursor ACC at the concentration of 20 - 50 μM could strongly enhance leaf explants to direct formation of embryos. We also found that both the ethylene inhibitors $AgNO_3$ and $CoCl_2$ significantly retarded direct embryo formation. Cameron *et al.* (1979) reported that ethylene production in plant tissue increase following an application of ACC. The suggestion is that ethylene may essential for the induction of direct somatic embryogenesis. However, ACC could promote or retard direct somatic embryogenesis from *Oncidium* leaf explants depending on the concentration. Lower concentrations (5 - 10 μM) of ACC not affect the embryogenic response of leaf tips and adaxial leaf regions, but retarded direct embryo formation from cut ends. However, higher concentrations (20 - 50 μM) strongly promoted direct embryo formation from leaf tips and adaxial leaf regions, but not affect embryo production on cut ends.

Although our experiments showed that the ethylene inhibitors retard direct somatic embryogenesis, the two inhibitors gave somewhat different results. They gave almost the same embryogenic response on leaf tips and adaxial leaf regions at lower concentrations (0.1 - 0.5 μM), but $CoCl_2$ resulted in more embryogenic response in comparison with $AgNO_3$ on cut ends. In addition, more embryo numbers per embryo-producing explants could be obtained on $CoCl_2$-containing medium. At higher concentration (5 μM), the embryogenic response of leaf tips and adaxial leaf regions was totally retarded by $AgNO_3$, but there were still 12.5 % of leaf regions formed embryos in the present of $CoCl_2$. However, $AgNO_3$ gave more embryogenic response than $CoCl_2$ on cut ends. $AgNO_3$ inhibit ethylene action through Ag^{2+} ions reducing the receptor capacity to bind ethylene, while $CoCl_2$ inhibit ethylene biosynthesis (Yang 1985). Thus, the different performances generated by the ethylene inhibitors tested may be a result of their different way of ethylene inhibition.

In a previous report, we found that TDZ was the most effective cytokinin to promote direct embryo formation from *Oncidum* leaf cultures (Chen *et al.* 1999; Chen and Chang, 2000b). Here we demonstrated that ACC alone was more effective than combination of TDZ and ACC on embryo numbers per embryo-producing explant.

In this study, we have shown that the application of ACC could stimulate embryogenic response of *Oncidium* leaf explants, but $AgNO_3$ and $CoCl_2$ mostly retarded it.

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