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ARTICLE

Indirect shoot organogenesis and plantlets regeneration from stem of ornamental *Dieffenbachia maculata* cv. Marianna

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ABSTRACT **The present study reports a simple protocol for indirect shoot organogenesis and plantlets regeneration of** *Dieffenbachia maculata* **cv. Marianna using stem segments from in vitro shoot culture. Different concentrations and combinations of indole-3-acetic acid (IAA), 1-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2, 4-D), 6-benzyladenine (BA), and kinetin (Kin) were used for callus induction and shoot organogenesis. The frequency of callus formation reached 87% and the highest number of shoots per callus was 4.8 for explants cultured on Murashige and Skoog (1962) medium supplemented with 15 mg l'[1] BA + 15 mg l'[1] NAA. Shoots were further grown and rooted on MS hormone free medium. Regenerated plantlets were successfully acclimatized in greenhouse with 100% survival rate.**
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KEY WORDS

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Dieffenbachia is a genus of tropical plants in the family Araceae noted for their patterned colorful, large leaves. The cells of the Dieffenbachia plant contain needle-shaped calcium oxalate crystals called raphides. If a leaf is chewed, these crystals cause a burning sensation in the mouth and throat, swelling can occur along with a temporary inability to speak and from this effect the plants are commonly called dumb cane. Dieffenbachia is often referred to as the "King of Plants". Members of this genus are extremely common houseplants because of their tolerance to shade. The genus has about 30 species, but the most widely grown cultivars are selections from *D. maculata* and *D. amoena.*

Vegetative propagation of *Dieffenbachia* is difficult, therefore, seedling propagation does not encourage the expansion of the species, moreover, seed production is limited (Henny 1988). In vitro methods of propagation are used for production of ornamental plants to meet the growing demand in both the domestic and the export market. Despite the increasing commercial demand of *Dieffenbachia* plants, only a few protocols for *in vitro* propagation through shoot cultures are published (Knauss 1976; Chase et al. 1981; Voyiatzi and Voyiatzis 1989; Henny et al. 2000; El-Mahrouk et al. 2006).

With the commercial application of *in vitro* propagation of *Dieffenbachia,* new cultivars have been released following selection of somaclonal variants (Chen and Henny 2006). The frequency of somaclonal variants is generally high, and the time required for a new cultivar release can be only 2 to 3 years compared to 7 to 10 years required using traditional breeding methods (Chen et al. 2003; Henny and Chen 2003). Successful use of *in vitro* techniques for producing somaclonal variants depends on the establishment of an efficient method for regenerating a large number of plants indirectly from an intervening callus stage (Arce-Montoya and Rodriguez-Alvarez 2006; Hammerschlag et al. 2006). Recently, indirect shoot organogenesis has been reported in *Dieffenbachia* cv. Camouflage using leaf explants (Shen et al. 2007). The objective of this study was to establish a protocol for inducing indirect shoot organogenesis in *Dieffenbachia maculata* cv. Marianna using stem segments.

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Materials and Methods

Plant material

In vitro plantlets of *Dieffenbachia maculata cv.* Marianna were maintained on MS (Murashige and Skoog 1962) solid medium (30 *g* l^{1} sucrose + 8.0 *g* l^{1} agar with 8.0 mg l^{1} BA) and kept at 25°C and 40 pmol m'^{2} s^{1} photosynthetic photon flux (PPF) (16 h/d) for *4* weeks. Shoots were individually separated and cultured on MS medium without growth regulators. After 3 weeks of culture, those shoots of about 3.0 cm in length were used for the experiments (El-Mahrouk et al. 2006)

Callus induction and shoot organogenesis

Stems obtained from the *in vitro* shoot cultures served as explant sources for callus induction. Stem explants were cut

Table **1.** Effect of IAA, NAA, 2,4-D on callus Induction of *Dieffenbachia maculata* cv. Marianna after 10 weeks in culture.

Auxins concentration (mg l^{-1})		Callus formation %	Callus diameter (mm)	Callus fresh weight (mg)
Auxin free medium		44.3 d[z]	42 bed	98 be
IAA	3	85.3 c	50 ab	105 be
	5	84.2 c	55 a	107 be
	10	85.2 c	54 a	154 a
	15	84.4 c	46 abc	114b
NAA	3	80.8 c	36 cd	80 be
	5	90.2 b	35 d	88 be
	10	93.2 b	44 bed	93 be
	15	91.6 b	54 a	170 a
2,4-D	0.1	98.1 a	40 bed	88 be
	0.5	90.4 b	34d	63 c
	1	84.3 c	43 bed	77 be
	3	0e	0 e	0d

[z] Mean separation within rows by Duncan's multiple range test at 5% level

Table 2. Effect of BA, Kin on callus Induction and shoot organogenesis of *Dieffenbachia maculata* cv. Marianna after 10 weeks in culture.

Cytokinin concentration (mg l^{-1})		Callus fresh weight (mg)	Callus diameter (mm)	Shoot organogenesis %	Number of shoots/ callus
Cytokinin free medium		100 c[z]	42 c	0d	0d
BA	5	1671 ab	67 b	50 b	2.1 b
	10	1323 ab	62 b	35 b	3.0 a
	15	998 b	65 b	72 a	3.1 a
	20	1902 a	89 a	3d	0.3 d
kin	5	1114ab	78 ab	38 b	1.7 c
	10	1174 ab	58 be	38 b	3.5 a
	15	1484 ab	58 be	37 b	3.3 a
	20	834 be	65 b	17 c	0.7 cd

[z] Mean separation within rows by Duncan's multiple range test at 5% level

into 5 mm^2 segments and cultured on MS medium with 3% (w/v) sucrose. A series of experiments were conducted using different auxins at different concentrations as follow: 2,4-Dicholorophenoxyacetic acid (2,4-D) at 0.1, 0.5, 1.0 and 2.0 mg l^{-1}; indole-3-acetic acid (IAA) and 1-naphthalene acetic acid (NAA) at 0, 3, 5, 10 and 15 mg l^{-1}; different cytokinins as benzyladenine (BA) and kinetin at concentrations of 0, 5, 10, 15 and 20 mg l^{-1} and different cytokinin/auxin combinations. Plant growth regulator-free medium served as the control. The pH of the medium was adjusted to 5.8 before autoclaving (at 121 °C and 1.2 kg cm^{-2} pressure for 15 min). Explants were cultured in 100 x 15 mm sterile glass Petri dishes containing 20 ml medium. There were 5 explants per Petri dish and *4* replicates per treatment. Cultures were maintained in dark for 8 weeks and *2* weeks in light at 40 pmol m^{-2} s^{-1} PPF. The

Table 3. Effect of BA, IAA, NAA on callus Induction and shoot organogenesis of *Dieffenbachia maculata* cv. Marianna after 10 weeks in culture.

PGRs concentration (mg l^{-1})	Callus fresh weight (mg)	Callus diameter (mm)	Shoot organogenesis %	Number of shoots/ callus
BA 15	976a[z]	95 a	73 b	2.8 b
BA15 + IAA 10	932 a	87 a	60 b	2.8 b
BA15 + NAA 10	980 a	103 a	87 a	4.8 a
BA 15 + IAA 10+ NAA 15	1048 a	105 a	61 b	2.0 b

[z]Mean separation within rows by Duncan's multiple range test at 5% level

callus fresh weight, callus diameter, number of shoots formed per callus, and callus formation as % of total number of explants, were recorded after 10 weeks of culture.

Development of shoot growth and rooting

Dieffenbachia shoots were separated and cultured on MS medium without hormones for their subsequent growth and rooting. The cultures were kept at 25°C and 40 pmol m^{-2} s^{-1} PPF (16 h/d) for *4* weeks.

Acclimatization

Plantlets at the 3-4 leaf stage were transplanted into culture pots (coffee cups) hlled with sterilized peat moss. The plantlets were grown in growth chamber for 1 week before their transfer to the greenhouse. The environment in the growth chamber was adjusted to a 25 ± 2°C air temperature, 40 - 50% relative humidity and a 100 pmol m^{-2} s^{-1} PPF with a 16 h photoperiod using halide lamps.

Experimental design and data analysis

Experiments were set up in a completely randomized design and repeated twice. Data were subjected to Duncan's multiple range test using SAS program (Version 6.12, SAS Institute Inc., Cary, USA).

Results and Discussion

Stem segments were cultured on MS medium supplemented with various auxins (IAA, NAA and 2,4-D) at different concentrations to determine the optimal auxin concentration for callus induction and growth (Table 1). The percentage of callus formation signihcantly increased at all auxin concentrations tested except that of 2.0 mg l^{-1} 2,4-D in comparison with auxin-free medium (the control). The highest percentage of callus formation was observed on medium containing 0.1 mg l^{-1} 2,4-D. The percentage of callus formation was signihcantly suppressed on medium supplemented with more than 0.1 mg l^{-1} 2,4-D. However, all concentrations of 2,4-D did not promote callus growth in terms of callus diameter and

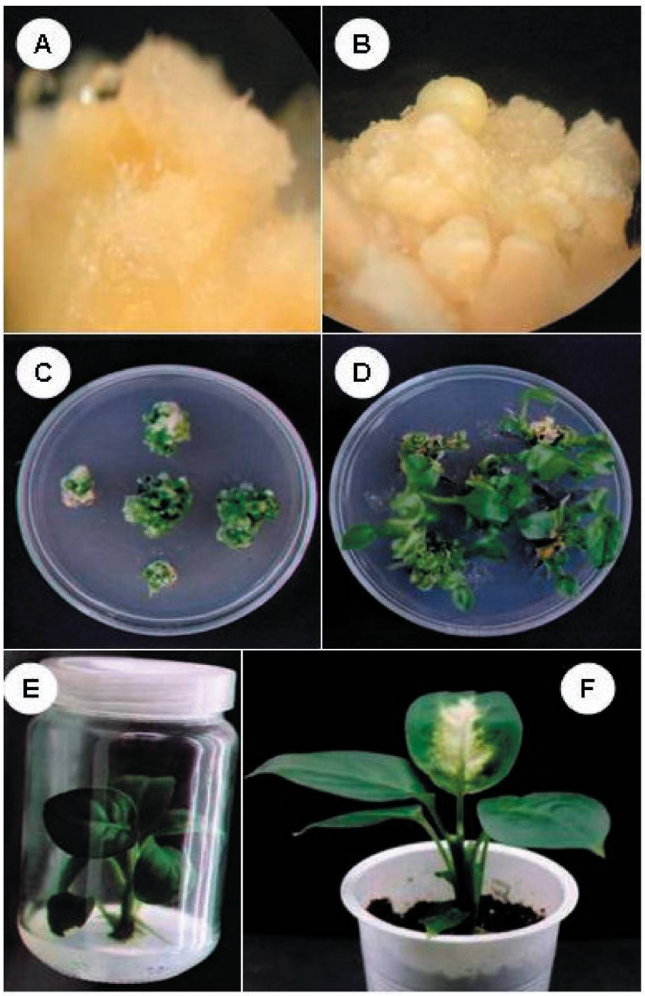

Figure 1. Indirect shoot organogenesis in Dieffenbachia. A, callus formation from stem segments; B, organogenic callus after 6 weeks of culture; C, D, greenish organogenic clusters forming shoots after 10 weeks; E, plantlets rooting in vitro; F, acclimatized plants in the greenhouse.

callus fresh weight. 2,4-D has been proved effective for callus induction in many plant species. In the present work, 2,4-D was not effective for callus growth and was not a prerequisite for callus induction. Similar results were also observed in *Dieffenbachia* cv. Camouflage (Shen et al. 2007). The callus formation observed on auxin-free medium indicates that the explants contained endogenous auxins which were enough to initiate callus. The best response for callus formation %, callus diameter and callus fresh weight was observed on medium containing 15 mg l^{1-} NAA as well as 10 mg l^{1} IAA. The calli were then transferred to MS medium containing different cytokinin types and concentrations and/or MS medium without growth regulators for *4* weeks (data not presented). No features of somatic embryogenesis or shoot organogenesis could be observed.

Callus induction and shoot organogenesis were investigated by testing cytokinins (BA, kin) alone. Shoot organogenesis responses caused by the various concentrations of cytokinins are presented in Table *2.* Proliferation responses were influenced by the cytokinin types and their concentrations. The highest shoot organogenesis percentage (72%) and number of shoots per callus (3.1) were obtained when the culture medium was supplemented with 15 mg l^{1} BA. Also, high shoot number per callus was observed when the culture medium was supplemented with 10 or 15 mg 1^{1} kinetin. However, the shoot organogenesis percentage was low at all the concentrations tested of kinetin. The type and concentrations of cytokinins are the key factors influencing indirect shoot organogenesis in *D. maculata* cv. Marianna. Variation in the activity of different cytokinins may be explained by their different uptake rate reported in different genomes (Blakesley 1991).

When BA was employed with NAA, the number of shoots per callus increased in comparison with treatments with BA alone (Table 3). In general, there were no significant differences in fresh weight and callus diameter at all tested combinations of 15 mg l^{1} BA with NAA or IBA. However, the largest number of shoots, 4.8 per callus, was obtained when the supplied auxin was NAA (10 mg l^{1}) combined with 15 mg 1^{-1}BA. The proportion of auxin-cytokinin is a determinant for meristem formation and the hormone balance that becomes established between growth regulators determines the type of buds induced (George 1993). The inherent endogenous auxin and cytokinin levels must have also played a role in the observed data (Pierik 1987). It is clear from our data that high cytokinin concentration is the main factor influencing indirect shoot organogenesis in *D. maculata* cv. Marianna. Application of NAA in combination with BA increased the number of shoots per callus. These data suggest that one mode of action for auxins could be to down-regulate both local cytokinin synthesis and cytokinin export from medium; this might influence the endogenous cytokinin levels and lead to the activation of buds (Eklof et al. 1997; Sato and Mori 2001).

Nodular calli were observed on the stem segments after 6 weeks of culture on MS medium supplemented with 15 mg l^{1} BA and 10 mg l^{1} NAA (Fig. 1 A, B). Small green meristems were visible on the surface of calli upon their transfer to light conditions. These meristems were later developed into shoot buds upon their subculture to MS medium hormone-free (Fig. 1 C). Shoot clusters with leaves were developed by the end of 10 weeks of culture (Fig. 1 D). The regenerated shoots via indirect shoot organogenesis were separated and cultured for *4* weeks on MS medium hormone-free for their rooting and 100% of plantlets were rooted (Fig. 1 E). *Dieffenbachia* plantlets (3-5 cm) at the 3-4 leaf stage were then grown in growth chamber for 1 week before their transfer to the greenhouse. The plantlets were acclimatized successfully (Fig. 1 F) with 100% survival rate.

In conclusion, the present study reported a simple protocol for in vitro production of *Dieffenbachia* plants via indirect shoot organogenesis. This protocol can be useful in further studies for screening somaclonal variations and developing new cultivars of this important ornamental plant.

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