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Plant Regeneration from Leaf Explants of *Kalanchoe daigremontiana* Hamet & Perrier

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ABSTRACT : Optimum culture conditions for high frequency plant regeneration from leaf explants of *Kalanchoe daigremontiana* Hamet & Perrier were established. Shoot regeneration was achieved from leaf explant cultures using MS medium supplemented with indole-3-acetic acid (IAA) and thidiazuron (TDZ) or benzyladenine (BA). Percent regeneration was influenced by plant growth regulators and source of explants. MS medium supplemented with TDZ (1.0 ㎎/ℓ) and IAA (0.4 ㎎/ℓ) was the most effective, providing shoot regeneration for 76.7 % of *ex vitro* leaf explants associated with a high number of shoots per explant (9.5 mean shoots per explant), whereas 100% shoot regeneration associated with 12.4 shoots per explant occurred from *in vitro* leaf explants on the same medium. Clusters of shoots were multiplied and elongated on MS medium containing several concentrations of BA. MS medium supplemented with 0.25 ㎎/ℓ BA was proved as the most effective shoot elongation medium. Elongated shoots (2-3 ㎝) were rooted at 100% on half-strength MS medium. Rooted plantlets were then transferred to potting soil. Regenerated plants were established in the soil with 90% success.

Key words : Crassulaceae, *In Vitro*, *Kalanchoe daigremontiana*, medium combination, plant growth regulators

INTRODUCTION

Kalanchoe daigremontiana Hamet & Perrier (Crassulaceae) is a succulent plant species, commonly known as 'mother of thousands', reproduces asexually by forming plantlets in leaf indentations (Warden, 1970a,b). It is widely distributed in tropical and subtropical countries and used in not only for landscape, garden, indoor decorations but also traditional medicine for the treatment of infections, rheumatism, cough, fever and inflammation (Hutapea, 1994). The fourteen sterols (Kalinowska *et al.*, 1990) and some insecticidal bufadienolides (Wagner *et al.*, 1985, 1986; Capon *et al.*, 1986) were isolated from the *K. daigremontiana*.

There are few previous reports of *in vitro* regeneration of *Kalanchoe* species. Smith and Nightingale (1979) reported regeneration of *Kalanchoe blossfeldiana* for the first time, achieving fast propagation of high quality plants. Bhuiyan *et al.* (2005) reported the mode of organogenesis of epiphyllous bud in *K. daigremontiana* during *in vitro* culture system. McKenzie *et al.* (1994) used *K. blossfeldiana* for transformation as a model species for the investigation of the *IPT* gene from *Agrobacterium*. Aida and Shibata (1996) also transformed *K. blossfeldiana*, and used it to study gene silencing patterns. Jia *et al.* (1989) reported high frequency transformation of *K. laciniata*. Mercuri *et al.* (1997) also stated transformation of *K. beharensis* using *Agrobacterium*. Up to now, the efficient regeneration system of *K. daigremontiana* has not yet been reported.

The objective of this study was to develop an efficient *in vitro* regeneration protocol as a necessary first step for the genetic transformation of molecular farming through valuable genes insertion into *Kalanchoe* species.

MATERIALS AND METHODS

Plant material

Stock plants of *K. daigremontiana* were grown in the greenhouse. The second or third pair of leaves from the top of the plant was excised and surface sterilized in 70% ethanol for 30 sec, washed in sterile distilled water and then soaked in 10% (v/v) commercial bleach solution containing a few drops of Tween-20 for 10 min with intermittent shaking. Surface-sterilized leaves were washed twice with sterile distilled water to avoid further tissue damage (Lee *et al.*, 1979). Sterilization

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often caused wounded parts of the leaf to change color from fresh green to whitish. Such whitish tissues were considered dead, and were cut off prior to prepare 5 × 5 ㎟ explants.

Media preparation and culture conditions

The basal medium consisted of Murashige and Skoog (1962) minerals and vitamins (MS), supplemented with 3.0% (w/v) sucrose, 0.1% (w/v) myo-inositol and 0.8% (w/v) agar (Maxim Bio Co. Ltd., Korea). This medium was supplemented with various combinations of the plant growth regulators (PGR) thidiazuron (TDZ) or benzyladenine (BA) and indole-3-acetic acid (IAA). The pH of all media was adjusted to 5.7 prior to addition of agar and sterilization at 121 ℃ for 15 min. Plant growth regulators (filter sterilized) were added after sterilization to media cooled to 55 ℃. Regeneration experiments were carried out using 90 × 15 ㎜ petri-dishes containing 25 ㎖ of medium. After positioning explants, petri-dishes were sealed with para film. Cultures were incubated at 25 ±2 ℃ with a 16/8 h light/dark photoperiod and irradiance of 30 μmol/㎡·s at self level provided by cool white fluorescent tubes and subcultured every 21 days.

Percent shoot regeneration from *ex vitro* leaf explants in response to plant growth regulators, explant orientation and photoperiod regime

In this investigation, MS medium supplemented with TDZ or BA and IAA were used. For each factor investigated, treatments consisted of three petri-dishes with ten leaf discs (explants) per dish. Explants were placed with the adaxial surfaces in contact or abaxial surfaces in contact with the media. To investigate the influence of the photoperiod regime on shoot regeneration, cultures were incubated at 25 ± 2 ℃ with a 16/8 h , 8/16 h, 24/0 h and 0/24 h light/dark photoperiod and irradiance of 30 μmol/㎡ · s and subcultured every 21 days. Experiments were done only once. Regeneration percentages were determined 6 weeks after the experiments were started.

Regeneration from leaf explants grown in *ex vitro*

This experiment included nine treatments consisting of three levels of IAA (0.2, 0.4, 0.8 ㎎/ℓ) combined with three levels of TDZ (0.25, 0.50, 1.0 ㎎/ℓ) arranged in a 3 × 3 complete factorial design. Explants were placed with the adaxial surfaces in contact with the regeneration media. Cultures were incubated at 25 ± 2 ℃ with a 16/8 light/dark photoperiod and irradiance of 30 μmol/㎡ · s and subcultured every 21 days. There were three petri-dishes per treatment with ten explants per dish. Regeneration percentages were determined 6 weeks after the experiment was started.

Regeneration from leaf explants grown in *in vitro*

Young leaves were collected from *in vitro* grown *K. daigremontiana* plant (30 days old) and the leaf blades were aseptically excised and cut into 5 × 5 ㎟ explants. The excised explants were cultured on MS medium supplemented with 0.4 ㎎/ℓ IAA and 1.0 ㎎ l^{-1} TDZ based on the results from Previous experiment. Cultures were incubated at 25 ± 2 ℃ with a 16/8 light/dark photoperiod and irradiance of 30 μmol/㎡ · s and subcultured every 21 days. The percentage of shoot formation and number of shoots per explant were recorded after 6 weeks of culture.

Shoot elongation

Clusters of shoots and buds were excised from the explants and transferred on the MS medium supplemented with 0.1, 0.25 and 0.5 ㎎/ℓ BA for 4 weeks. A total of 10 clusters were used in each treatment for shoot elongation.

Rooting and acclimatization

Shoots (2~3 ㎝) were excised and placed on solid rooting medium in 130 × 60 ㎜ glass bottles. Each bottle contained 40 ㎖ medium with four shoots. The rooting medium contained half-strength MS minerals, full-strength MS vitamins, 3% (w/v) sucrose and 0.8% (w/v) agar. 20 shoots were used in this experiment and the experiment was duplicated. Cultures were incubated under light and temperature conditions similar to that used for shoot regeneration experiments. After 30 days in rooting media, the rooted plantlets were carefully extracted from the tissue culture vessels and washed gently with water to remove agar medium and sucrose traces to discourage infection by fungal contaminants. And then plantlets were planted into plastic pots containing soil. The pots were covered with a transparent plastic cup to ensure high humidity during the acclimatization period of 15 days. Acclimatized plants were then transferred to the green house.

Statistical analysis

For each treatment, 10 leaf explants were cultured on the petri-dishes and three plates were used for each treatment. Each plate was regarded as individual replicate and used for statistical analysis. For all experiments data were collected

Table 1. Effect of plant growth regulators on % shoot regeneration of *Kalanchoe daigramontiana* after 6 weeks in culture.

Plant growth regulators (㎎/ℓ)	Shoot formation (%)
IAA 0.4 + TDZ 0.5	53.3 ± 9.1
IAA 0.4 + BA 0.5	30 ± 8.4

Values represent means ± standard error.

Table 2. Effect of leaf disc orientation on % shoot regeneration of *Kalanchoe daigramontiana* on MS medium supplemented with 0.4 mg/ℓ IAA and 0.5 mg/ℓ TDZ after 6 weeks in culture.

Leaf disc orientation	Shoot formation (%)
Abaxial down	53.34 ± 9.1
Adaxial down	60 ± 8.9

Values represent means ± standard error.

Table 3. Effect of photoperiod regime on % shoot regeneration of *Kalanchoe daigramontiana* on MS medium supplemented with 0.4 mg/ℓ IAA and 0.5 mg/ℓ TDZ after 6 weeks in culture.

Photoperiod regime	Shoot formation (%)
16 h light + 8 h dark	66.67 ± 8.6
8 h light + 16 h dark	46.67 ± 9.1
24 h light (continuous light)	20 ± 7.3
24 h dark (continuous darkness)	16.67 ± 6.8

Values represent means ± standard error.

after six weeks of *in vitro* culture. For statistical analysis, percent data were transformed into arcsine values and analyzed by ANOVA, and means were subjected to LSD and ANOVA tests using SAS statistical package (SAS Institute Inc. 1985).

RESULTS

Percent shoot regeneration from *ex vitro* leaf explants in response to plant growth regulators, explant orientation and photoperiod regime

In preliminary studies, TDZ along with IAA at specific concentrations produced the highest rate of shoot regeneration in *Kalanchoe daigremontiana* than those of BA (Table 1). The regeneration percentages were high for explants placed as adaxial side down to the media compared to explants placed as abaxial side down to the media (Table 2). Shoot regeneration was higher in cultures incubated with a 16/8 h light/dark photoperiod as compared to those incubated in continuous light and darkness (Table 3).

Regeneration from leaf explants grown in *ex vitro*

Of the combinations, MS medium supplemented with TDZ (1.0 mg/ℓ) and IAA (0.4 mg/ℓ) was the most effective, providing shoot regeneration for 76.7% of explants associated with a high number of shoots per explant (9.5 mean shoots per explant) (Table 4). Explants grown in this medium for 3 weeks formed callus at the cut surface, and after 3 weeks the callus began to produce multiple shoot primordial, which developed into adventitious shoots (Fig. 1).

Table 4. Effect of different combinations of IAA and TDZ on shoot regeneration from ex *vitro* leaf explants of *Kalanchoe daigremontiana* after 6 weeks in culture.

Growth regulators (mg/ℓ)		% explants producing shoots	No. of shoots/ explant
IAA	TDZ		
0.2	0.25	10.0 ± 5.5e	6.2 ± 0.4b
	0.5	43.3 ± 9.0c	8.2 ± 0.5ab
	1.0	26.7 ± 8.1d	6.2 ± 0.4b
0.4	0.25	26.7 ± 8.1d	8.2 ± 0.5ab
	0.5	60.0 ± 8.9b	9.2 ± 0.1a
	1.0	76.7 ± 7.7a	9.5 ± 0.4a
0.8	0.25	16.7 ± 6.8e	3.6 ± 0.6c
	0.5	43.3 ± 9.0c	4.3 ± 0.7c
	1.0	16.7 ± 6.8e	5.7 ± 0.7b
MS basal		0	0

Values represent means ± standard error. Mean values within the column followed by the same letters were not significantly different at p = 0.05, LSD.

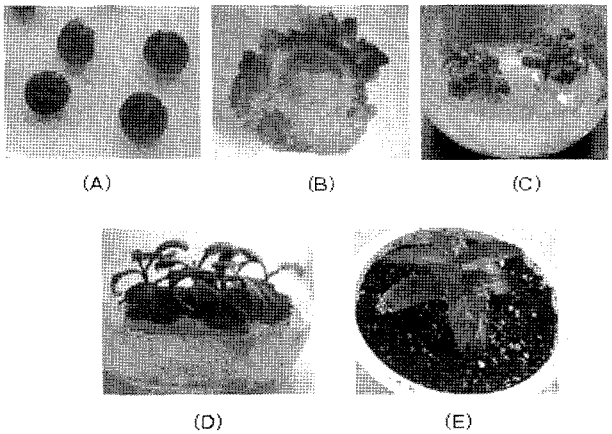

Fig. 1. Plant regeneration from ex *vitro* leaf explants of *Kalanchoe daigremontiana;* (A) Callus induction on MS medium supplemented with 0.4 mg/ℓ IAA and 1.0 mg/ℓ TDZ; (B) Multiple shoot induction on MS medium supplemented with 0.4 mg/ℓ IAA and 1.0 mg/ℓ TDZ; (C) Multiplication and elongation of shoots on MS + 0.25 mg/ℓ BA for 4 weeks; (D). Rooted plantlets on half-strength MS medium; (E) An acclimatized plant in pot soil.

Regeneration from leaf explants grown in *in vitro*

In this experiment it was also found that explant source significantly enhanced shoot organogenesis from leaf discs. 100% of *in vitro*-excised explants produced shoots associated with 12.4 mean shoots/explant, whereas only 76.7% *ex vitro* excised explants produced shoots on MS medium supplemented with 0.4 mg/ℓ IAA and 1.0 mg/ℓ TDZ (Fig. 2).

Shoot elongation

MS medium supplemented with TDZ produces highest rate

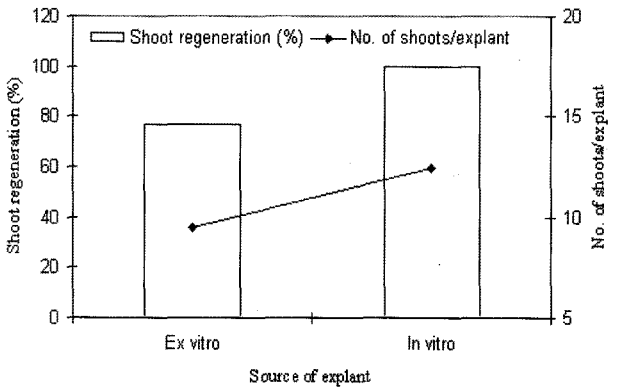

Fig. 2. Effect of explant source on shoot regeneration in MS medium supplemented with 0.4 mg/ℓ IAA and 1.0 mg/ℓ TDZ after 6 weeks in culture.

Table 5. Effect of various concentration of BA on shoot elongation of *Kalanchoe daigremontiana* after 4 weeks in culture.

Medium	Average shoot length (mm)	Highest shoot length (mm)
MS basal	6.2 ± 0.4c	10.13 ± 0.4c
MS + BA 0.1 mg/ℓ	12.0 ± 0.5b	15.1 ± 0.7b
MS + BA 0.25 mg/ℓ	24.8 ± 0.1a	31.0 ± 0.6a
MS + BA 0.50 mg/ℓ	11.2 ± 0.4b	13.0 ± 0.4b

Values represent means ± standard error. Mean values within the column followed by the same letters were not significantly different at p = 0.05, LSD.

of shoot organogenesis, but the shoots were very short to count. To stimulate elongation of the shoots and to obtain a favorable morphology, BA at different concentration (0.1, 0.25 and 0.5 mg/ℓ) was tested. All combinations led to a greater elongation of internodes than in control (plant growth regulator free medium). With increasing concentration of BA, shoot length was decreased (Table 5). Some rooted shoots were observed on hormone free medium or with BA at concentrations of 0.1 mg/ℓ. Shoots shorter than 5 mm was not counted, because they were too small to count and were considered not being able to produce plantlets.

Rooting and acclimatization

Root formation occurred as early as 10 days after placement of shoots in rooting medium (Fig. 1D). 100% of shoots were rooted. 90% of rooted plantlets acclimatized and grew vigorously with no appearance of morphological abnormalities.

DISCUSSION

The first morphogenetic change, observed after one week in culture, was the development of callus at the cut surfaces. The explants cultured on the medium without plant growth regulator turned brown or died in 3-4 weeks. Cell divisions started near the wounded edges. First growth activities of the leaf segment appeared at its lower side near to the agar medium's surface.

Shoot regeneration from leaf explants was highly variable depending on the plant growth regulators, leaf explant orientation and photoperiod regime. During the preliminary regeneration trials, the best results obtained on MS medium supplemented with 0.4 mg/ℓ IAA and 0.5 mg/ℓ TDZ, and explants placed as adaxial side down to the media under 16/8 h light/dark phptoperiod regime. Frello *et al.* (2002) reported that regeneration of *Kalanchoe* was higher in MS medium containing TDZ and IAA. TDZ was also found to be more effective than BA in the regeneration of other species such as *Kalanchoe laciniata* (Jia *et al.*, 1989), *Malus* (Fasolo *et al.*, 1989) and *Pyrus* (Chevreau and Skirvin, 1992). Born *et al.* (1994) reported that shoot regeneration was higher in long day (16 h light) conditions compared to short day (8 h light) and continuous dark conditions.

The results from media containing 0.4 mg/ℓ IAA indicate that increasing the level of TDZ to above 1.0 mg/ℓ might increase the number of explants producing shoots (Table 4). TDZ was more effective at promoting regeneration than BA. The biological activity of TDZ in many species is higher than or comparable to that of the most active adenine-type cytokinins (Moc *et al.*, 1987; Huetteman and Preece, 1993). Percentage of shoot regeneration was also influenced by explant sources. We found that *in vitro* -grown explants produce high rate (100%) of shoot regenetaion on MS media supplemented with 1.0 mg/ℓ TDZ and 0.4 mg/ℓ IAA whereas *ex vitro*-grown explants produce only 76.7% of shoot regeneration on the same media. This observation corresponds with the result of Schneider-Moldrickx *et al.* (1984). The high number of shoots per explant was also occurred from *in vitro*-grown explants (12.4 mean shoots/explant) as compared to *ex vitro*-grown explants (9.5 mean shoots/explant). The higher shoot regeneration rate of *in vitro*-grown explants might be due to younger tissue, still developing, having less differentiated and more metabolically active cells, with a more suitable hormonal and nutritional status, all of which could improve direct shoot organogenesis.

In our experiments, a good result obtained by using TDZ, but the regenerated shoots were usually bushy and small, and rooting them was difficult. Similar observation has also been described by Bates *et al.* (1992), Murthy *et al.* (1998) and Yusnita *et al.* (1990). Previous study revealed that BA was best cytokinin for shoot elongation and multiplication. So we used

BA in this study to elongate the regenerated shoots.

Generally, *in vitro*-shoot, root and callus production from various explants is known largely to be determined by the balance between concentrations and kind of auxins and cytokinins in the growth medium. This has been confirmed by our study. Summarising our results it can be asserted that a highly reproducible method for *K. daigremontiana* was established. Of the nine combinations, MS medium supplemented with 0.4 mg/ℓ^{-1} IAA and TDZ 1.0 mg/ℓ^{-1} was proved as the best medium for shoot regeneration of *K. daigremontiana.*

Recently, there are some reports to get medicinal components from herbs cultured *in vitro*, such as *Pelagonium* (Agarwal and Ranus RS, 2000; Hwang, 2006) and *Valeriana* (Li *et al.*, 2006). Our regeneration system for *K. daigremontiana* is a simple and efficient method. This system will be used for transformation experiments with plasmids carrying useful genes and for the efficient production of medicinal components from *K. daigremontiana.*

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