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In Vitro propagation of a woody ornamental *Eranthemum nervosum* (Vahl) R. Br.

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Various factors affecting culture establishment, shoot proliferation and rooting of *Eranthemum nervosum* (Vahl) R. Br., a woody shrub, were studied. Leaf explants were the most suitable explants for *in vitro* mass propagation via both organogenesis and embryogenesis. Induction via indirect organogenesis was done by culturing young leaves on Murashige and Skoog (MS) medium supplemented with 0.1 mg/l NAA and 1.0 mg/l 2, 4-D for callus proliferation, followed by 2 transfers onto a medium containing 0.1 mg/l NAA and 0.05 mg/l BAP at 6 weeks intervals. A four to five shoots/cluster was produced. Induction via direct organogenesis or embryogenesis was achieved when culturing explants on medium solidified with 0.8% agar or 0.15% phytagel, respectively. Somaclonal variation was observed from plantlet regeneration via indirect organogenesis. Rooted shoot was easily induced on MS or ½ MS medium solidified with both phytagel and agar-agar. Acclimatization on vermiculite gave 100% plantlet survival. Regenerated plants were successfully transferred to soil. Thus, following this protocol, it would be possible to produce more than 100,000 plants from a single leaf cutting in a year.

Key words: *Eranthemum nervosum* (Vahl) R. Br., woody ornamental, organogenesis, embryogenesis, somaclonal variation

Introduction

Eranthemum nervosum (Vahl) R. Br. (= *pulchellum*), blue sage or blue erthemum, a perennial branched shrub. The genus *Eranthemum*, belonging to the family Acanthaceae, comprising nearly 104 species. This genus is cultivated thoughout the tropics as an ornamental plant. These woody plants flower continuously and are relatively free from pest and disease. This plant is a popular garden ornamental flower because of the rich bright gentian violet flower, which occurs in terminal spikes and the plant also has attractive foliage (Gilman, 1999). Since, the flowers look like those of the genus *Ixora* that belongs to the family Rubiaceae, *E. nervosum* is called Violet Ixora in Thailand. *E. nervosum* is used

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extensively in plant masses and herbaceous borders. All of the cultivated species are propagated from softwood cutting. The rate of multiplication using softwood cutting is not sufficient for commercial planting. In addition, this method is labor intensive and expensive. Thus, tissue culture techniques may be useful for the mass propagation of this plant. Many plant species such as gardenia (Al-Juboory *et al*., 1998), apple (Fasolo *et al*., 1989), ash gourd (Thomas and Sreejesh, 2004), chilli paper (Kumar *et al*., 2005) and lotus (Arunyanart and Chaitrayagun, 2005) were successfully propagated through this technique. Although, successful application of tissue culture technique with herbaceous species have been shown to have limitations for woody species. Several studies have been carried out to optimize conditions for the *in vitro* regeneration and multiplication of woody species such as *Ixora coccinea* (Lakshmanan *et al*., 1997), *Feronia limonia* L. (Hiregouder *et al*., 2003), *Feijoa sellowiana* (Stefanello *et al*., 2005) and sweet sherry (Bhagwat and Lane, 2004). However, efficient and wildly applicable *in vitro* propagation systems have not yet been developed for *E. nervosum*. This work was undertaken to study the effect of different explant types, plant growth regulators (PGR) and gelling agents on mass propagation in *E. nervosum.*

Materials and methods

Plant material and culturing conditions

Seeds of *E. nervosum* from the garden of Prince of Songkla University, Hat Yai, Songkla, Thailand, were surface sterilized with 70% (v/v) ethanol for 1 min, followed by disinfecting in 20% Clorox for 15 min before being rinsed three times in sterile distilled water. The treated seeds were germinated on MS (Murashige and Skoog, 1962) basal medium containing 3% (w/v) sucrose and 0.8% agar, pH 5.8. Culture media was autoclaved at 120 °C for 20 min. Cultures were incubated at 25±2 °C under 16-h photoperiod using cool, white florescent light (ca 1,500 lx) until germination.

Effect of plant growth regulators on explants proliferation

Shoot tip, cotyledon, immature leaf, stem and proximal root excised from 2-week-old plant were cultured on MS medium supplemented with various concentration of NAA (0, 0.1, 0.25 and 0.5 mg/l) in combination with BA (0.1, 0.5, and 1.0 mg/l) for preliminary study of explant proliferation. The results were evaluated after 4 weeks of culture. For callus induction, pale, immature and mature leaf and callus were cultured on MS medium supplemented with 0.1 mg/l NAA in combination with 1.0 or 3.0 mg/l 2, 4-D. For shoot induction, 4 week-old proliferated friable callus were transferred to culture medium containing BAP in

combination with NAA at 0.1 and 0.5 mg/l. Shoot multiplication was achieved by culturing 1 cm-shoots on MS medium supplemented with 0.1 mg/l NAA in combination with BAP or KN at 0.05 and 1.0 mg/l for 4 weeks before evaluated.

Effect of gelling agent on explants proliferation

Immature and mature leaf (4 -week-old), stem, shoot and proximal root were cultured on medium supplemented with 0.1 mg/l NAA, 1.0 mg/l BA and solidified with 0.8% agar or 0.2% phytagel for 4 weeks before evaluated.

Root induction and acclimatization

Shoots (3-4 cm long) were excised individually and transferred to rooting medium. The medium was hormone-free full or half strength MS supplemented with 3% sucrose and solidified with 0.2% phytagel or 0.8% agar. Complete plantlets, 5-6 cm in length, were gently washed in tap water to remove agar from their roots and were then transferred to plastic cups (upper diameter 6 cm x length 8 cm) filled with vermiculite or garden soil mixed with rice ash, organic fertilizer and coconut fiber (2:1:1:1). For acclimatization, plastic cups were kept under high humidity in plastic boxes that coved with a lid and irrigated once a day. After 4 weeks, the plants were transferred to larger pots containing garden soil and grown in a greenhouse. The plants were irrigated with fertilizer (15 N: 15 P: 15 K; Brand Raeu-Bai, Thailand, 1kg/6.25 US$, concentration 1g/l) at 2-week intervals. After 2 months, plantlets were established.

Experimental design and analysis of data

All experiments employed a completely randomized design (CRD). Each treatment consisted of 4 replications and each replication had 40 explants. Data presented are mean ± standard error. Data were evaluated by one way analysis of variance (AOVA) and comparisons between the mean value of treatment made by Duncan's multiple range test (DMRT) using IRRISTAT (International Rice Research Institute, version 92-1).

Results

Effect of plant growth regulators on explants proliferation

The preliminary study on the effect of plant growth regulators on explants proliferation found that NAA and BAP had a positive effect on explants proliferation. Leaf explants dramatically responded to NAA and BAP-

supplemented medium more than the others. Immature leaves cultured on medium containing 0.1 mg/l NAA and 1.0 mg/l BAP enlarged and developed 4-5 shoots directly at the cut surfaces within 4 weeks. The induced shoots grew and elongated very well with dark green leaves. Occasionally, lower or higher NAA and BAP-supplemented medium resulted in callus formation with no shoot proliferation. Shoot tips cultured on 0.1 or 0.5 mg/l BAP-supplemented medium in combination with 0.1 mg/l NAA resulted in single shoot production. Increasing the NAA and BAP concentration promoted callus proliferation with no further development. Cotyledons did not respond very well in medium supplemented with 0.25 mg/l NAA with all combinations of BAP. Lower or higher NAA in the presence of BAP induced cotyledons to callus profusely. However after the end of culture, the inducing calli turned brown. Similar to stems, the presence of NAA and BAP-supplemented medium induced calli with no proliferation. Moreover, NAA and BAP-supplemented medium failed to show any response on root explant proliferation.

For callus induction, medium containing 1 mg/l 2, 4-D gave higher percentage of callus induction and multiplication. Increasing of 2, 4-D concentration up to 3 mg/l inhibited and slowed the induction. Additionally, explants showed a different response to 2, 4-D. Immature leaves gave the highest percentage of callus induction (97.78%±3.85%) where as pale leaves gave the lowest percentage of callus induction (77.96±33.23%) (Table 1). Moreover, 2, 4-D at 1.0 mg/l promoted the multiplication of callus up to 93.33±11.55%. Calli proliferated from all of the explants after treatment on this medium for 2 weeks. All of the induced callus proliferated from the cut surfaces and subsequently covered the entire surface of the explant after culture for 4 weeks. The initiated calli were white and friable which grew rapidly into green compact callus upon subsequent culture on medium with 0.1 mg/l NAA and 0.05 mg/l BAP for shoot induction (Table 2). These callus directly formed shoot primordia via organogenesis of 4-5 shoots, after subculture on the same medium for 4 weeks. The regenerated shoot show further elongation on this medium and a maximum shoot length of 0.5-1 cm was observed. Higher NAA-concentrations inhibited the percentage of shoot induction and shoot number.

Effect of gelling agent on explants proliferation

Gelling agent had a significant effect on explants proliferation. Agar-medium trended to promote shoot induction via direct organogenesis whereas phytagel-medium promoted various types on explant proliferation. Cultured stem-explants on agar-medium gave the highest percentage of shoot induction (100%) via direct organogenesis. Similar results were observed in shoot tip,

proximal root and immature leaf cultures. Among these cultures, proximal roots gave the lowest percentage of shoot induction and numbers of shoots. However, this explant when cultured on phytagel-medium showed an ability to produce multiple shoot via direct organogenesis with 8-9 shoots per explant. On the contrary, stems and shoot tips cultured in phytagel-solidified medium yielded callus induction. In the case of immature leaf culture, shoots were induced via both direct organogenesis (Fig. 1A) and embryogenesis (Fig. 1B). The percentage of direct organogenesis induction and number of shoot were higher than somatic embryogenesis induction.

Table 1. Effect of 2, 4-D and explants on callus induction after culture on MS medium for 4 weeks.

Growth regulator (mg/l)		% Callus formation (mean±SE)			
		Explants			
NAA	**2,4-D**	**Pale leaf**	**Mature leaf**	**Immature leaf**	**callus**
0.1	1	77.96±33.73a*	88.41±8.52a	97.78±3.85a	93.33±11.55a
0.1	3	15.72±11.19b	10.82±9.57b	60.34±4.15b	46.67±7.22b

*Mean having the same letter in a column was not significantly different by Duncan's Multiple Range Test, $P \leq 0.05$

Table 2. Effect of the ratio of NAA to BAP on shoot proliferation after culture on MS medium for 4 weeks.

Growth regulator (mg/l)		% shoot induction (mean±SE)	no. of shoots(mean±SE)
BAP	**NAA**		
0.1	0.05	72.72±3.52a*	4.40±0.65ns
0.5	0.05	50.73±4.65b	3.68±0.57

*Mean having the same letter in a column was not significantly different by Duncan's multiple range test, $P \leq 0.05$

ns = not significantly different by Duncan's multiple range test, $P \leq 0.05$

Table 3. Effect of BAP and KN on multiple shoot induction after culture on MS medium for 4 weeks.

Growth regulator (mg/l)		% shoot induction (mean±SE)	no. of shoots (mean±SE)	% root formation (mean±SE)	No. of roots (mean±SE)
NAA	BAP				
0.1	0.05	25.28±1.34c*	2.45±1.59a	62.51±0.79a	1.43±0.56b
0.1	0.1	51.41±2.39a	2.64±1.42a	25.59±3.49c	1.38±0.62b
NAA	KN				
0.1	0.05	37.50±1.45b	1.54±0.43b	50.75±1.14b	3.24±0.87a
0.1	0.1	25.46±2.78c	1.67±0.29b	62.53±3.56a	2.32±0.34a

*Mean having the same letter in a column was not significantly different by Duncan's multiple range test, $P \leq 0.05$

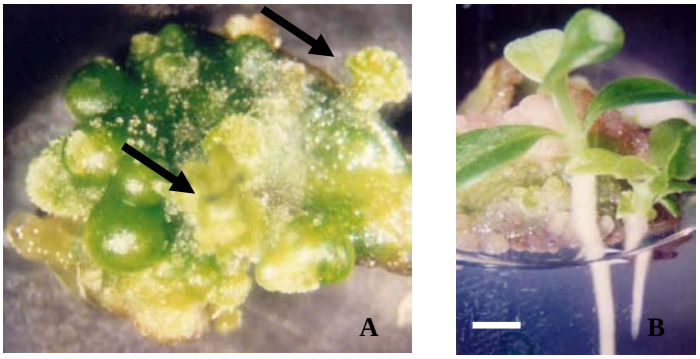

Fig. 1. Proliferation of leaf explant cultured on phytagel-medium. Bar=0.1 cm. (A) shoot induction via direct organogenesis. (B) plantlet induction via embryogenesis.

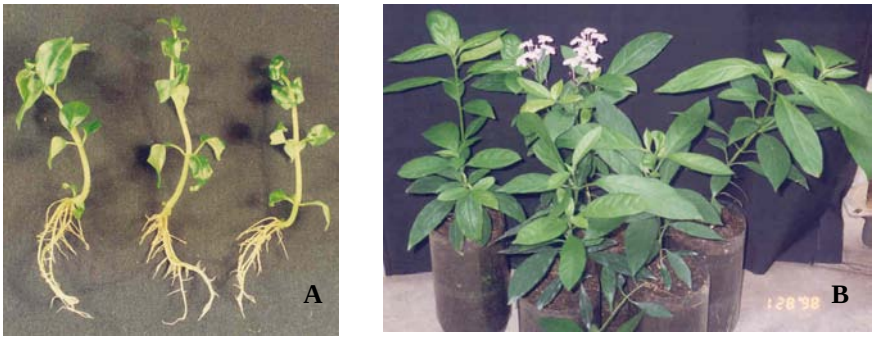

Fig. 2. Plantlet induction. (A) Plantlet with healthy root. Bar=0.5 cm (B) 2-months plant with first flower. Bar = 10 cm.

Rooting and acclimatization

There were no significant differences among treatments for root induction. Medium concentrations and solidifying agents stimulated 100% root induction after 2 weeks of culture. Also, the number of roots (2.93±0.53) and the root length (3.77±0.38 cm in length) showed no significant. The emerged roots from all experiments were healthy with a white color (Fig. 2A). Moreover, root hairs were observed on the primary roots. For acclimatization, two-week-old plantlets grown in vermiculite had a higher percentage survival rate (98.45±3.47%) than in garden soil (65.52±4.82%) (Table 4). One-month plantlets, 15-cm tall were transferred to larger poly-bags filled with garden soil and watered twice a day. After 2 months, the plants were fully developed (ca. 50 cm height) and flowering suitable for commercial sole (Fig. 2B).

Table 4. Effect of soil material on planted acclimatization after culture for 4 weeks.

Soil material	% plantlet survival (mean±SE)
Vermiculite	98.45±3.47a*
garden soil	65.52±4.82b

*Mean having the same letter in a column was not significantly different by Duncan's multiple range test, $P \leq 0.05$

Discussion

To our knowledge this work is the first report of a simple and reproducible protocol for the *in vitro* mass propagation of *E. nervosum* via organogenesis and embryogenesis. Most of the reports on *in vitro* mass propagation of woody ornamental plants describe either organogenesis (Jain *et al.*, 2002; Hiregouder *et al*., 2003; Thomas and Sreejesh, 2004; Stefanello *et al.*, 2005) or embryogenesis (Kintzios *et al*., 2000; Pareek and Kothari, 2003). In this study, an efficient procedure for continuous, high frequency shoot multiplication and plantlet production of *E. nervosum in vitro* was developed. The results showed that the immature leaf was the most suitable explant for *in vitro* culture. Immature leaves showed a higher percentage of callus induction and plantlet regeneration both via organogenesis and embryogenesis. Yamanouchi *et al*. (1999) also reported stable regeneration of plants from immature leaves of mulberry. This difference in proliferation capacity is generally known that different explant sources differ in their morphogenic capability.

Also, the plant growth regulator had an important role on explant proliferation. In this study, NAA in combination with BA was found to be essential for proliferation of the explants. Similar results were reported in ash gourd (Thomas and Sreejesh, 2004) and cloudberry (Martinussen *et al*., 2004). For indirect organogenesis induction, it was found that the 2, 4-D concentration markedly affected the rate of calli induction. Higher 2, 4-D concentrations inhibited callus formation. Furthermore, better callus proliferation was observed in immature rather than mature leaves. This is similar to the results of callus induction in immature and mature leaf explants of *Phlox paniculata* Linn. (Jain *et al*., 2002). Callus when transferred to culture on BAP-medium for 6 weeks developed shoots. However, variation occurred from the callus mediated regeneration. Normally, *E. nervosum* have an elliptic leaf shape with an erect one shoot (Gilman, 1999), but in this study, three types of leaf shape and three types of stem shape were seen. This finding agrees with D'Amato (1975) who reported that regeneration from an intermittent callus phase is not advantageous and causes genetic variability. Martinussen *et al*. (2004) also

reported that *in vitro* propagation can lead to somaclonal variation and changes in the phenotypes, especially if high concentrations of plant growth regulators are used in the medium. Furthermore, it was found that BAP was the most effective cytokinin compared to KN for multiple shoots production. Similarly, results are reported in mulberry (Pattnaik *et al*., 1996) and *Feronia limonia* Linn. (Hiregouder *et al*., 2003).

For direct organogenesis and embryogenesis, it was surprising that the gelling agent can play a role on proliferation. Agar-medium trended to induce direct shoot organogenesis without callus phase, whereas phytagel-medium had more effect on proliferation. Embryo-derived plants are found as the edge of the explant whereas direct shoot formation occurred on an abaxial site up on the leaf. The result obtained in this study was similar to Pareek and Kothari (2003) who found that direct somatic embryogenesis from leaf explants of *Dianthus* occured from the cut edges of leaf explants. It was possible due to the changes at the level of endogenous growth regulators (Ivanova *et al*., 2004). Moreover, it was found that NAA at 0.1 mg/l in combination with BAP at 1.0 mg/l had the greatest influence on plantlet induction. Similar observations on embryogenesis potential subjected to high levels of cytokinin and low levels of auxin were reported in *Typhinium trilobatum* (Das *et al*., 1999) and *Cephaeli sipecacuanha* (Rout *et al*., 2002). The opposite result was found in *Dianthus* species, where 2, 4-D alone is significant and indicated an important hormone in somatic embryogenesis (Pareek and Kothari, 2003).

For induction of roots, shoots normally from roots when treated in either MS or half strength MS medium without hormones. Normally, the rooting of shoots occurred on low salt MS hormone free medium or in combination with IBA or NAA. The use of low salt MS medium for rooting of the *in vitro* induced shoots is a very common practice (Khalafalla and Hattori, 1999).

Conclusions

The results obtained in the present work showed that immature leaf explant was the most suitable for *in vitro* mass propagation of *E. nervosuma* through three pathways: indirect organogenesis, direct organogenesis and embryogenesis. These young leaves when cultured on MS medium supplemented with 0.1 mg/l NAA, 1.0 mg/l BAP and solidified with 0.8 g/l agar or 0.15% phytagel for 4 weeks resulted in shoot induction or plantlet regeneration, respectively. For indirect organogenesis, cultured this explant on medium supplemented with 0.1 mg/l NAA and 1.0 mg/l 2, 4-D for 4 weeks resulted in callus production. These calli continuous cultured on medium containing 0.1 mg/l NAA and 0.05 mg/l BAP for 6 weeks with 2 subcultures promoted multiple shoots induction. For root induction, shoots were induced

on MS or ½ MS medium-free hormone and solidified with either 0.8 g/l agar or 0.15% phytagel. Transferring plantlets to vermiculite for 4 weeks, gave the highest percentage of plantlet survival. Thus, the success in raising *E. nervosum* plantlets through micro-propagation has opened up the possibility for large-scale clonal propagation. With this protocol described here, more than 100,000 plants could be produced from a single leaf cutting in a year, compared with a few plants produced by conventional methods (by softwood cuttings) during the same period. We believe that this protocol could be adapted for the propagation of other *Eranthemum* species as well as other woody species of economic importance. Also, the ability to regenerate shoots *in vitro* is useful directly for genetic transformation research, as well as for plant improvement via somaclonal variation.

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