Micropropagation of *Euphorbia Milii* Desmoul *via* Cyathium Explants

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Key words: *Euphorbia milii*, Inflorescence, Plantlets

Summary

Micropropagation of *Euphorbia milii* Desmoul 'FBR' was successful by using inflorescences as explants source. The first stage of cyathium cultured on one and half strength of MS medium containing 5 mg/l BA was the most effective on vegetative shoot regeneration. The vegetative shoots obtained from cyathium *in vitro* were used as explants for multiplication. The higher numbers of multiple shoots were achieved with shoots subcultured on full strength of MS medium containing 2 mg/l BA. There were more shoots when subcultured on semi-solid medium, while shoots were hyperhydricity when shoots were subcultured on support with liquid medium. For *ex vitro* rooting, shoots were harvested from *in vitro* culture and cut into single shoot of 1.5 cm in length and treated with auxin powder. *E. milii* microcutting rooted well when treated with 4 g/Kg IAA or NAA. However, microcuttings treated with 1 g/Kg IAA, 2 g/Kg IBA or control got 100% of survival rate after transplanting for 2 weeks. Moreover, there were found plantlets propagated by micropropagation had more branches than conventional cuttings.

Introduction

Euphorbia milii Desmoul, is a member of Euphorbiaceae, Spurge family. It is a popular easy-care indoor flowering pot plant (McLaughlin and Garofalo, 2002). In 2003, there were 640,133 plants of *E. milii* both with and without flowers exported from Thailand. In 2005, the volume of exported *E. milii* increased to 1,079,172 plants as well as the value was 17,191,210

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Baht. The major markets were Philippines, the Netherlands, and Indonesia (Dibanuka, 2006).

Normally, *E. milii* is easily propagated by cutting, grafting, and seeding, but sensitive to fungal problems during propagation (http://www.tropicanursery.com/euphorbia/pdfs /crown of thorns makes a comeback.pdf). Furthermore, these methods produced low progeny numbers (Dewir *et al*., 2005).

Plant tissue culture was very useful to rapid mass production (Topoonyanont *et al.,* 1999; Airo *et al*., 2007). In addition, micropropagated plants were uniform, compact and to develop more basal shoots than cuttings (Chu, 1991). *In vitro* studies on the species of *Euphorbia* had been reported. However, those plants had endogenous bacteria infected in vascular tissue, and toxic exudates were common problems (Ripley and Preece, 1986). For example, shoot tip of *E. milii* had been used as initial explant, that was difficult to obtain contaminate-free plant material *in vitro* (Dewir *et al*., 2005).

The potential of inflorescence explants for micropropagation in several plant species had been recognized, such as *Limonium* (Topoonyanont *et al*., 2000), *E. milii* (Dewir *et al*., 2005), and *Petasites hybridus,* (Wildi *et al*., 1998). The advantages of using inflorescence as initial explants were easy for sterilization and the mother plant was not destroyed (Dewir *et al*., 2005). However, there was little information about *E. milii* micropropagation for commercial. In this research, an efficient micropropagation was developed.

Materials and Methods

Materials

Euphorbia milii 'FBR', a free branching type with small red bract, grown in greenhouse of National Chung Hsing University, was used as initial explant source. The cyathium developmental was grouped into 3 stages according to the opening of male flowers (stamens) and female flower (pistil) presented. At the 1st stage, immature bracts unfolded. At the 2nd stage, bracts began to open and there was female flower growing up. At the 3rd stage, bracts opened fully and there were male flowers growing up (Fig. 1).

Methods

1. Initial culture

The cyathia were surface sterilized with 1% sodium hypochlorite for 8 min followed by 3 times rinse with sterile distilled water. Then the peduncle was cut into 1 mm in length. The sterilized cyathium of 1st stage was incubated individually in each test tube which filled with 10

ml semi-solid medium containing full strength Murashige and Skoog (1962) basal medium, 30 g/l sucrose, and supplemented with benzyladenine (BA) at 0, 1, 5, 10, 15, or 20 mg/l, respectively. Or the MS medium at ¼, ½, 1 or 1½ MS strength formula with 5 mg/l BA was used. Moreover, sterilized cyathia of 1st, 2nd, or 3rd were cultured on semi-solid medium containing full strength MS with 5 mg/l BA.

After 1 month of culture, the explants were developed into vegetative shoots, intermediate shoots (vegetative and floral), floral shoots, or none. The characteristic of vegetative shoots were noticed as a shoot with leaves, floral shoots were developed into inflorescences, while inter-mediate developed to both vegetative and reproductive shoots (Fig. 2)

Fig. 1. Cyathium stage of *E. milii* 'FBR' grouped into 3 stages; 1st: immature bracts unfolded, 2nd: bracts began to open and there was female flower growing up or 3rd stage: bracts opened fully and there were male flowers growing up, respectively.

Fig. 2. Cyathium explants of *E. milii* 'FBR' developed into reproductive (left), intermediate (middle), and vegetative shoot (right), respectively.

2. Subculture

(i) Shoot multiplication in semi-solid culture

New shoots approx. 1 cm long from initial cultures were subcultured in GA-7 vessel (Magenta Corporation, Chicago, IL. U.S.A). Five shoots were incubated in each vessel filled with 50 ml semi-solid medium half strength MS medium containing 1 mg/l NAA and combination with BA at 0, 1, 2, or 4 mg/l, respectively. MS medium at the strength of 1, ½, or ¼ with 2 mg/l BA and combination with 1 mg/l naphthalene acetic acid (NAA) were tested, respectively. In the experiment of BA and MS effect, each treatment had 5 replications, while in experiment of auxin effect.

(ii) Shoot multiplication on media of different phases:

Multiplication medium, containing half strength of MS salts, 30 g/l sucrose, 2 mg/l BA and 0.01 mg/l NAA, or without auxin was used for culturing *E. milii* 'RBG'. Explants were cultured on semi-solid medium or GROW CUBES (Pioneer U.N. Co., Ltd.) used as supporting material in liquid culture. Five shoots (approx. 1 cm long) from *in vitro* cultures were incubated in a GA-7 vessel.

All shoot multiplication data were collected after 6 weeks of subculture in terms of multiplication rate, shoot number in different shoot height (grouped into lower than 0.5 cm, 0.5-1.0, 1.1-1.5, 1.6-2.0 and more than 2.1 cm), percentage of hyperhydric shoots, fresh and dry weight, and callus in diameter. Dry weight was determined by drying shoots in a forced air oven for 48 hours at 60°C. Each treatment had 5 replications.

All semi-solid media were solidified with 7 mg/l agar, except in media for initial culture 6 mg/l agar was used. The pH value was adjusted to 5.7 before autoclaving at 121°C for 15 min. *In vitro* cultures were incubated at 25±2°C under a 16 h day-length with photosynthetic photon flux density (PPFD) of 35 μmol $m^{-2}s^{-1}$, provided by fluorescent tubes (Taiwan Toshiba FL 40D/30).

3. *Ex vitro* rooting

Shoots were harvested from *in vitro* culture and cut into single shoot of 1.5 cm in length under non-aseptic condition. The basal part of shoot was powdered with rooting powder containing indoleacetic acid (IAA), indolebutyric acid (IBA), or NAA at 0, 1, 2, or 4 g/Kg. In other test, shoots were cut into different sizes of 1, 1.5 or 2 cm in length and was powdered with rooting powder containing 2 g/Kg IBA. Then, these microcuttings were inserted into 128-cell-tray filled with growing medium (1:1; Bas Van Buuren (BVB) No. 4 medium, Visser Co., the Netherlands, and perlite No. 4, South Sea Trading Co., Ltd. Taiwan). After 6 weeks, all microcuttings were collected data. Each treatment had 10 microcuttings.

Growth and rooting responses were colleted in terms of shoot height, number of leaves,

width of the widest leaf, length of the longest leaf, number of roots, percentage of rooting, length of the longest root, number of flowers, and callus in diameter.

Plants propagated by conventional cutting and micropropagation were planted in a 7 cm pot which filled with growing medium. After 2 months, the performance of pot flower was compared in the terms of stem length, number of shoot per pot, number of flowers per, plantlet diameter, and leaf area. The leaf area was measured by using leaf area software written by Dr. Shen-Lin, Lin, Horticulture department of National Chung Hsing University.

4. Experimental design

All experiments were repeated 3 times and set up in completely randomized design (CRD). Data from all variables were analyzed by analysis of variance (ANOVA) and treatment means were separated using Fisher's Least Significant difference (LSD) test at $p = 0.05$ (Ott, 1988).

Results

1. Factors to reversion of *E. milii* 'FBR' cyathia

When cyathium explants were cultured on MS medium containing BA at 0, 1, 5, 10, 15, or 20 mg/l, the more vegetative shoots were obtained on the medium containing 1 mg/l BA, the next was cultured on the medium containing 10 or 5 mg/l BA. Cyathium cultured on medium containing 20 mg/l BA regenerated more intermediate shoots, but fewer vegetative shoots. The cyathium developed more reproductive shoots when it was cultured on medium containing 0 or 1 mg/l BA. However, over half of explants cultured on the medium without BA did not grow any shoot (Fig. 3).

The strength of MS medium enhanced shoots growth from cyathium explants (Fig. 4). The more vegetative shoots developed when cyathium cultured on medium containing higher strength of MS from ¼ to 1½. But the higher intermediate shoots developed from cyathium on half strength of MS medium. On the cultured of full strength of MS, there were more developed reproductive shoots. When cyathium was cultured on medium containing ¼ strength of MS, over 60% of cyathium did not regenerate any new shoot (Fig. 4).

There were 24%, 36%, or 4% of cyathium explants at the 1st stage developed vegetative shoots, intermediate shoots, or reproductive shoots, respectively, when cyathium were cultured on MS medium containing 5 mg/l BA. On contrary, less than 10% of cyathium explants at 2nd stage developed vegetative shoots, but more than 30% of the 2nd cyathium developed reproductive shoots. However, there was no shoot developed when the cyathium at 3rd stage were cultured (Fig. 5).

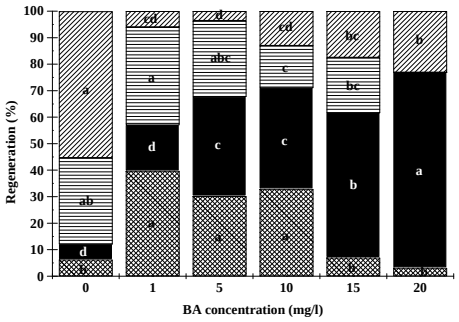

Fig. 3. Effect of BA on regeneration of cyathium explant (%) of *E. milii* 'FBR' after 30 days of initial culture. ▨ vegetative shoot; ■ intermediate shoot; ▤ reproductive shoot; ▨ no development.

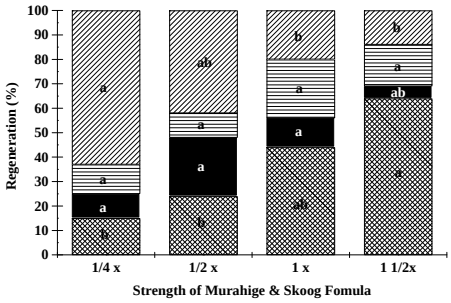

Fig. 4: Effect of MS strength on regeneration of cyathium explant (%) of *E. milii* 'FBR' after 30 days of initial culture. ▨ vegetative shoot; ■ intermediate shoot; ▤ reproductive shoot; ▨ no development.

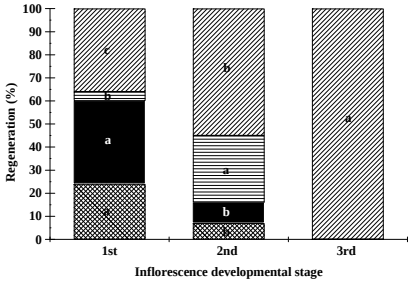

Fig. 5. Effect of cyathium stage on regeneration of *E. milii* 'FBR' cultured on MS medium supplemented with 5 mg/l BA after 30 days of initial culture. ▨ vegetative shoot; ■ intermediate shoot; ▤ reproductive shoot; ▨ no development.

2. Shoot multiplication on semi-solid culture

Shoots were subcultured on MS medium containing BA at 0, 1, 2 or 4 mg/l, respectively. The increasing BA concentration enhanced small shoots development. There were more short shoots (< 0.5 cm in height) developed on medium supplemented with 2 or 4 mg/l BA, while larger multiple shoots developed from shoots cultured on medium supplemented with 1 mg/l BA (Fig. 6).

The strength of MS in medium also had influenced on multiplication of shoots. The higher strength of MS medium produced the more and larger shoots. Shoot subcultured on full strength of MS medium proliferated more shoots over 1 cm in length and over 2 cm in length. On contrary, shoot subcultured on MS medium at ¼ or ½ strength did not produced any shoot larger than 1.5 cm (Fig. 7).

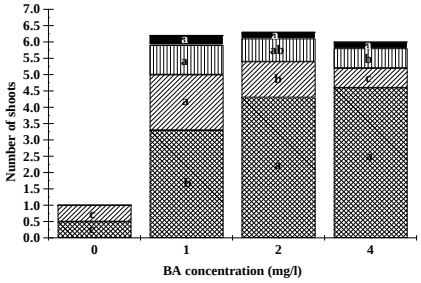

Fig. 6. Effect of BA on multiple shoots in of *E. milii* 'FBR' after 6 weeks of subculture. Shoots grouped as <0.5 cm; 0.5-1.0 cm; 1.1-1.5 cm; 1.6-2.0 cm

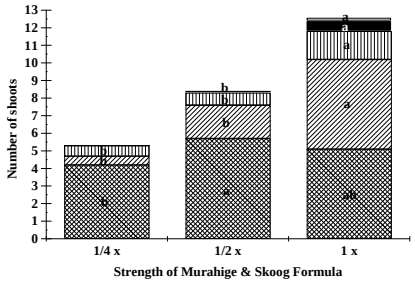

Fig. 7. Strength effect of Murashige & Skoog formula on multiple shoots of *E. milii* 'FBR' after 6 weeks of subculture. Shoots grouped as <0.5 cm; 0.5-1.0 cm; 1.1-1.5 cm; 1.6-2.0 cm; over 2.1 cm.

3. Shoot multiplication on different medium phase

The results clearly indicated shoots grown on semi-solid medium produced more shoots than those grown on support with liquid medium (Fig. 8). The greater fresh and dry weight and callus in diameter occurred in semi-solid culture. However, large shoots over 1.6 cm in length were observed only in liquid culture. In addition, there were occurred hyperhydric shoot when cultured on liquid culture.

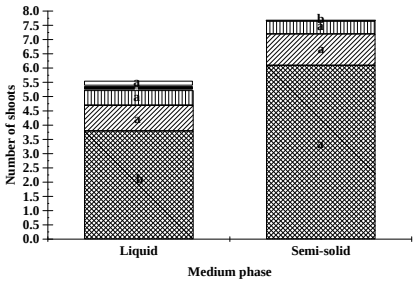

Fig. 8. Effect of medium phase on multiple shoots of *E. milii* 'FBR' after 6 weeks of subculture. Shoots grouped as <0.5 cm; 0.5-1.0 cm; 1.1-1.5 cm; 1.6-2.0 cm; over 2.1 cm.

4. *Ex vitro* rooting

The microcuttings rooted *ex vitro* after treated with IAA, IBA, or NAA rooting powder at 0, 1, 2, or 4 g/Kg, respectively (Table 1). Every shoot after treated with auxin powder was rooted better than control, except those treated with 1 g/Kg IAA. The higher number of roots were observed on microcutting powdered by 4 g/Kg IAA or NAA. Whereas, the plantlets with longer roots were found on microcuttings treated with IBA. But there was no significant difference to compare with treatment of control or 2 g/Kg IAA. The plantlets had more leaves when they were treated by 2 g/Kg IBA. Overall, IAA promoted shoots with wider and longer leaves when IAA concentration was decreased from 4 to 1 g/Kg. Although, those shoots were rooted well, but only plantlets developed from shoots powdered by 1 g/Kg IAA, 2 g/Kg IBA or control got 100% of survival rate after transplanting for 2 weeks (Table 1).

Table 1. Effect of auxin on microcutting growth of *E. milii* 'FBR' after rooting *ex vitro* for 6 weeks.

Auxin	Conc. (g/Kg)	Rooting (%)	No. of roots/shoot	Length of the longest root (cm)	Callus diameter (cm)	Shoot height (cm)	No. of leaves/ shoot	Width of the widest leaf (cm)	Length of the longest leaf (cm)	Survival rate (%)[z]
Control	0	96a[y]	7.7d	4.5ab	0.3ab	2.1ab	7.7abcd	0.8cd	1.59de	100a
IAA	1	93a	10.8bcd	3.8bcd	0.3ab	2.2a	7.9abc	1.0a	2.23a	100a
	2	100a	12.3bcd	4.0abcd	0.4a	2.2a	7.8abcd	0.9ab	2.10ab	93abc
	4	100a	14.8ab	3.8bcde	0.3ab	2.0ab	7.6bcd	0.8abc	1.91abcd	87bc
IBA	1	100a	9.5cd	4.3abc	0.3ab	2.1a	8.0ab	0.9abc	2.01abc	93abc
	2	100a	12.3bcd	4.7a	0.4a	1.9ab	9.1a	1.0ab	2.16a	100a
	4	98a	10.1bcd	3.9abcd	0.3ab	2.0ab	7.0bcd	0.8bcd	1.79bcde	93abc
NAA	1	100a	10.4bcd	3.6cde	0.3b	1.8b	6.4d	0.8bcd	1.70cde	83c
	2	100a	13.8bc	3.1e	0.4ab	1.9ab	6.4cd	0.7d	1.50e	83c
	4	96a	19.9a	3.4de	0.4ab	2.2a	7.9abc	0.9abc	1.97abc	97ab

[z] Survival rate was record after 2 weeks of transplant.

[y] Means separation within each column followed by LSD test at $P \leq 0.05$.

5. Performance of potted *E. milii* 'FBR'

There was no significant differences in the stem length, plantlet diameter and leaf area between plantlets propagation by conventional cutting and micropropagation (Table 2). But the plantlets propagation by micropropagation had more shoots than plantlets propagation by cutting had. However, the florets of plantlets propagation by micropropagation were less than that of plantlets propagation by cutting in 2 months.

Table 2. The effect of propagation method on the performance of potted *E. milii* 'FBR' after transplanting into 7 cm pot for 2 months.

Propagation method	Stem length (cm/pot)	No. of shoot/pot	No. of floret/pot	Plantlet diameter (cm)	Leaf Area (cm^2/pot)
Conventional cutting	11.0a[z]	5b	22a	13.7a	126a
Micropropagation	9.9a	11a	10b	13.0a	131a

[z] Means separation within each column followed by LSD test at $P \leq 0.05$. Data collected after transplanting for 2 months.

Discussion

Usually, the floral organs are the upper part of plant. There are sepals or bracts to protect them from insect feeding and to keep bacterial and fungal spores away (Mauseth, 2003). Thus, they had advantages for establishing *in vitro* culture, such as ease of sterilization, low contaminations, and conservation of the mother plant utilized to initial culture (Singh and Sehgal, 1999; Dewir *et al*., 2005). In addition, at tip of young inflorescence, the development of buds was indeterminated. Therefore, inflorescences had been recognized as potent floral organs source for micropropagation of several plant species. For example, *Ocimum sanctum* Linn. (Singh and Sehgal, 1999), *Limonium* (Topoonyanont *et al*., 2000), *Phalaenopsis* (Tanaka and Sakanishi *et al*., 1987), and *E. milii* (Dewir, 2005). There were many reports showed the developmental stage and level of inflorescence had influence on regeneration or reversion of inflorescence. Topoonyanont *et al*. (2000) found that the young or early stage of *Limonium* inflorescence was more effective on reversion. In our studies, supported these findings since the higher percentage of reversion occurred when cyathium explant at first stage (Fig. 5) was used

than those at advanced stages.

The cyathium at first stage was indeterminated, while at 2nd and 3rd stage were determinated, that developed visible floral organs arranged in whorls. Because of the *E. milii* had patterns of reproductive development as in the first indeterminated vegetative meristems will precede transform into inflorescence meristems, and then regenerate into determinant floral meristems (Fosket, 1994).

Cytokinins were the major factor on shoots proliferation. Heloir *et al*. (1997) had reported, the medium containing BA only was optimum for multiplication of grapevine. In apple culture, the result was the same (Lane, 1978). Although some crops were observed that BA, with a low level of auxin was necessary for optimum proliferation as *Rosa damascena* Mill. (Jabbarzadeh and Khosh-Khui, 2005). However, when BA concentration was increased, shoot length of multiple shoots was reduced. Such as, *Achras sapota* (Purohit and Singhvi, 1998), *Gerbera jamesonii* (Aswath and Choudhry, 2005), and begonia (Nakano *et al*., 1999). Fig. 4 shows that not only the multiple shoots were enhanced, but shoots shorter than 0.5 cm long also were enhanced when BA concentration was increased from 0 to 4 mg/l. In some cases, high BA could lead to the abnormality of growth as fasciations in *Prunus avium* (Kitin *et al*., 2005), multi-apex in strawberry (Anderson *et al*., 1982), and bushiness in gerbera (Topoonyanont *et al*., 1999). In this experiment, *Euphorbia milii* 'FBR' had abnormal shoots as fasciations of *Prunus avium* when subcultured on medium containing BA at 5 mg/l for long time. However, some abnormal shoots could revert to normal shoots when subcultured on hormone free medium.

Murashige and Skoog's (1962) medium had been used for micropropagation in several plants. The strength of MS had influenced on growth during multiplication. Tomsome *et al*. (2007) found shoot length of *Syringa vulgaris* L. was enhanced when the strength of MS macrosalt in medium was increased from 1 to 1.5 times. While Pierik *et al*. (1988) found the optimal strength of MS for the shoot length or multiplication rate of *Syringa vulgaris* L. was optimal at 1 or 1.25 times, respectively. And the percentage of leaf curling was decreased when MS strength was increased from 0.5 to 1.5 times and completely disappeared at 1.5 or over times. However, the shoot length and multiplication rate were strongly declined when that MS strength was over than 1.25 times. On the contrast, the best shoot growth and multiplication rate of *Gerbera hydrida* Hort. was obtained on half strength of MS medium (Huang and Chu, 1985). In this experiment, the full strength of MS medium was more effective on shoot growth and multiplication of *Euphorbia milii* (Fig. 7).

The liquid medium could enhance growth and multiplication rate of several plants more than solid medium in micropropagation. For example chrysanthemum (Hahn and Paek, 2005), pineapple (*Ananas comosus* L. Merr) (Escalona *et al*., 1999), and plantain (*Musa* AAB) (Roels

et al., 2005). However, Debergh *et al*. (1992) found liquid medium could induce hyperhydricity tissue of explant material. In this experiment *E. milii* was observed hyperhydricity shoots when cultured on liquid medium with support. Moreover, shoots cultured on semi-solid medium had higher multiple shoots than on liquid medium. But the shoots longer than 1.6 cm was observed on liquid medium only (Fig. 8)

Hatzilazarou *et al*. (2003) reported both *in vitro* and *ex vitro* rooting of *Bougainvillea* sp. 'Alexandra' microcutting rooted well when it was treated with IAA. Moreover, both under *in vitro* and *ex vitro* condition IBA had been widely used as rooting hormone, such as wild yams (Poornima and Ravishankar Rai, 2007), *Drosophyllum lusitanicum* (Gonçalves and Romano, 2007), or *Nerium oleander* (Hatzilazarou *et al*., 2003). In present study, we found 2 g/Kg IBA powder was the most effective rooting hormone for of *E. milii*. The survival rate in this experiment was high to 100% (Table 1).

Our results were show *E. milii* 'FBR' plantlet propagation by micropropagation had more shoots that was very compact than that propagation by cutting. But they had a few flowers (Table 2). Probably, plantlets propagation by micropropagation was more juvenile those than that propagation by cutting.

Gonçalves and Romano (2007) had been reported the *ex vitro* rooting of *D. lusitanicum* favors was a simple step with reduction of micropropagation cost. The combination of tissue culture and *ex vitro* rooting of microcutting was suitable for commercial production of plantlets of *E. milii.*

In conclusion, *Euphorbia milii* micropropagated by using cyathium explants at first stage cultured on one and half strength of MS medium containing 5 mg/l BA was the most effective on reversion into vegetative shoots. The vegetative shoot proliferated more shoots on semi-solid media containing full strength of MS with 2 mg/l BA. Microcuttings 1.5 cm in length was powdered by 2 g/Kg IBA powder. Following these cheaper plantlets and better pot flower of *E. milii* were produced.

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麒麟花利用花序培殖體之微體繁殖

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關鍵字：麒麟花、花序、種苗

摘要：以易分枝型(free-branching)之紅花麒麟花(*Euphorbia milii* Desmoul 'FBR'),品種為材料，進行微體繁殖。將第一期大戟花序培養在含5 mg/l BA的1又1/2 MS培養基中，可得較多的再生芽體。此外麒麟花之芽體分別繼代培養在含2 mg/l BA之MS培養基中，芽體增殖數較多。芽體培養在固體培養基的增殖芽體數較培養在液體培養的多，芽體在液體培養時會有玻璃質化的現象。瓶外發根實驗時，由組培苗獲得的芽體切成1.5cm長的單芽，再沾取含有auxin的粉劑。處理4g/Kg的IAA或NAA時，麒麟花微插穗的發根情形良好。然而微插穗移植兩週後，處理1 g/Kg的IAA、2g/Kg的IBA或對照組可以得到100%的存活率。比較利用傳統的扦插或微體繁殖的苗株上盆2個月的盆花，微體繁殖的苗株上盆後分枝數較扦插苗多，但花朵數較少。

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