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Research Article

Direct androgenesis in anther culture of butterfly pea related to flower development

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Abstract

Androgenesis is a biotechnological pathway of *in vitro* gametic embryogenesis with the potential leading to haploid plant production from male reproductive organ. Butterfly pea (*Clitoria ternatea* L.) is a versatile leguminous plant with the floral shape resembling a butterfly at full bloom. Based on the varying lengths of flower as well as petals and sepals, 5 stages of flower development were delineated. When anthers from flowers of these various stages were cultured on basal Murashige and Skoog medium supplemented with 3 mg/l 2,4-D and 60 or 80 g/l sucrose, it was found that anther-derived embryos (11.67 and 3.3% in the presence of 6 and 8% sucrose, respectively) were obtained only from stage 2 flowers. Light microscopic observation also revealed that the microspores of anthers isolated from flowers at stage 2 was uninucleate. This suggested that anther or microspore stage of butterfly pea played a very important role in direct androgenesis and 8% sucrose in the medium was detrimental to this process.

Keywords: androgenic embryo, anther, butterfly pea flower, microspore

Introduction

Butterfly pea (*Clitoria ternatea* L.), belongs to the sub-family Papilionaceae in Fabaceae, and is a nutritive multi-purpose forage legume originated from tropical Asia. This perennial edible leguminous twiner may be used as a nutraceutical, medicinal or ornamental plant. Besides, it is also valuable in cuisine and for rotational fodder (Gomez & Kalamani, 2003; Barik et al., 2007; Morris, 2009).

Clonal propagation *in vitro* can potentially be achieved via an organogenic and embryogenic pathway. For embryogenesis, adventive or nonzygotic embryoes are induced either from somatic or gametic material. In gametic embryogenesis, androgenesis is a part of this pathway involving the development of embryos in cell culture initiated from isolated anther or microspore or immature pollen (Maraschin et al., 2005; Forster et al., 2007). Although there have been many publications on organogenesis and somatic embryogenesis of butterfly pea, there are few investigations of androgenesis of this crop (Barik et al., 2007; Nair & Reghunath, 2008; Mukhtar et al., 2012). In particular, the objective of this study was to examine the

relationship between flower developmental stages and anther-derived embryo formation in butterfly pea.

Materials and methods

Different sizes of butterfly pea flowers, blue flower with double petalloid cultivar, were collected (before blooming and at anthesis) at around 8-9 a.m. from the garden of the Division of Agro-Industrial Technology, Faculty of Applied Science, King Mongkut's University of Technology North Bangkok. Firstly, the lengths of the outermost petal, sepal and entire flower as well as flower width were measured. Subsequently, the various developmental stages were operationally classified based on the different sizes of the floral parts. Then, the length and width of the anther as well as the length of the filament at each developmental stage were also determined.

For gametic embryo induction, butterfly pea flower at various developmental stages were rinsed with sterilized distilled water for 5 min, surface disinfested by soaking for 15 min in 20% (v/v) Clorox household bleach containing 3 drops of Tween 20, followed by immersion in sterilized distilled water 3 times (1 min each). Then, the petals were excised and discarded while the stamens were placed on a sterile Petri dish. Afterward, the filament was excised from the anther before the anther was cut transversely and transferred to induction medium [MS (Murashige & Skoog, 1962) semi-solid medium supplemented with 3 mg/l 2,4-D and either 60 or 80 g/l sucrose]. Subsequently, the cultures were kept for 4 weeks in a culture room under 25^{o}C and 16 h of 20.87 μmol/m^{2}/s lighting daily. Afterwards, androgenic embryos formed were transferred to grow on basal MS medium containing 30 g/l sucrose.

To determine the developmental stages of the microspores, a procedure modified from Wang et al. (2010) was used. Two anthers dissected from a flower of an appropriate developmental stage were placed on a glass slide and then covered with another glass slide before a glass rod was rolled over the upper glass slide for 10 s. The upper glass slide was removed so that microspores could be treated with 2 drops of 40% (v/v) Clorox household bleach for 1 min, followed by adding 1 drop of distilled water and covered with a cover slip. Finally, a tissue paper was used to absorb any excess solution and then a drop of 1% (w/v) acetocarmine was added at one side of the cover slip to stain the microspores for 20 min before observation under a light microscope.

Results and discussion

Various sizes of butterfly pea flowers were collected for size measurements of the petals, sepals and the whole flowers so that 5 stages of flower development were identified based on statistically significant differences in the lengths of petals, sepals and flowers (Table 1, Figure 1A). Similarly, the filament also exhibited different lengths in relation to the 5 stages of flower development. This result is quite similar to the measurement of filament length in most species of *Collinsia* as the filament gradually elongates from the first until the final stage of flower development (Armbruster et al., 2002). However, there was little difference in the anther size in relation to the first 4 stages of flower development while at stage 5 the anther size was significantly reduced. This was an overall trend opposite to those of the other flower parts (Table 2, Figure 1B and 1C).

Table 1. Analysis of butterfly pea flowers, the petals and sepals to delineate 5 stages of flower development (data from 10 replications)

Flower stages	Petal length (cm)	Sepal length (cm)	Flower length (cm)	Flower width (cm)
1	0.34±0.07a	1.05±0.10a	1.27±0.15a	0.54±0.07a
2	0.57±0.07b	1.30±0.07b	1.68±0.04b	0.66±0.05b
3	1.03±0.09c	1.38±0.08c	1.99±0.09c	0.73±0.05b
4	1.44±0.11d	1.47±0.08d	2.46±0.16d	0.74±0.05b
5	2.88±0.09e	1.60±0.07e	3.99±0.17e	2.82±0.16c

Means±S.D. in the same column with different letters were statistically significant different (P≤0.05)

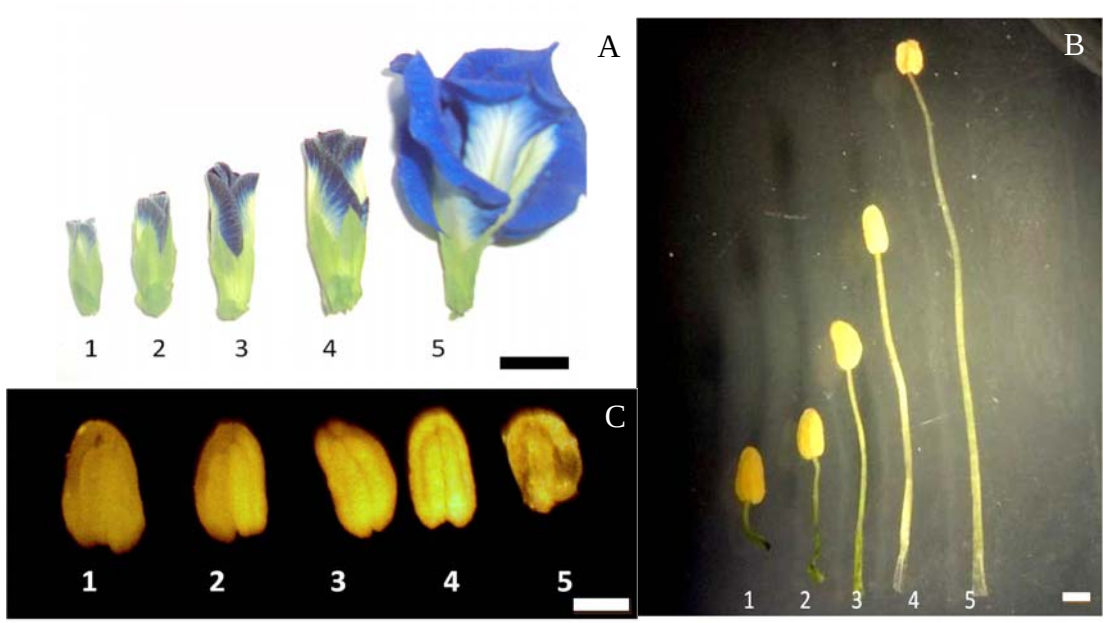

Figure 1. Butterfly pea: (A) flowers at various stages of development (scale bar = 1 cm), (B) stamens at various stages (scale bar = 0.1 cm), (C) anthers at various stages of flower development (scale bar = 0.1 cm)

After the different developmental stages were delineated, anthers from the flowers of the different stages were cultured on an androgenic embryo induction medium (see Materials and Methods). In preliminary experiments, it was found that there was no callus or gametic embryo formed when the entire butterfly pea anthers were cultured for 30 days. However, culturing the anther halves obtained by dissecting the anthers in the middle transversely resulted in formation of androgenic embryos. Dissection of anther was a kind of wounding or mechanical stress that stimulated androgenic embryo formation in some plants. For example, excision of anther tip was also found to enhance this process in white cabbage (Osolnik et al., 1993) and brussels sprout (Krzyżanowska & Górecka, 2008).

Table 2. Size of stamen at 5 stages of butterfly pea flower development (data from 30 replications)

Flower stages	Filament length (cm)	Anther length (cm)	Anther width (cm)
1	0.32±0.05a	0.20±0.02a	0.14±0.02a
2	0.46±0.06b	0.19±0.03ab	0.14±0.02a
3	0.74±0.09c	0.19±0.02ab	0.13±0.02ab
4	0.97±0.09d	0.18±0.03b	0.12±0.03b
5	1.84±0.10e	0.12±0.03c	0.11±0.02c

Means±S.D. in the same column with different letters were statistically significant different (P≤0.05)

The procedure used for androgenic embryo formation in this study was slightly adapted from a similar research of Sudhersan et al. (2008) on Sturt's desert pea (an ornamental legume which is like butterfly pea belonging to the family Fabaceae). Although the induction medium used gave the best percentage of embryogenic callus initiation in cultured Sturt's desert pea anthers, the response of butterfly pea anther explants on the same medium was dissimilar. It was found that androgenic embryos of butterfly pea developed directly from the cultured anthers (Figure 2A) and only anther explants from flowers of the stage 2 development could form this embryogenic structure. In addition, the number of anther-derived embryos in two treatments (each from 60 replications) decreased from 11.67±0.32 to 3.3±0.18% when sucrose concentration in the culture medium was increased from 60 to 80 g/l, respectively.

An examination under a light microscope revealed that the microspores from the anthers of flowers at stage 2 development were at the uninucleate stage (Figure 2B). It was, however, found that without exine removal according to the modified method of Wang et al. (2010), the nucleus of butterfly pea microspore was hardly to be seen. The uninucleate condition of the microspores might be important for androgenic embryo induction in butterfly pea. The same result was also found in other plants, such as peanut (Willcox et al., 1991) and chickpea (Panchangam et al., 2014), that are in the same family as butterfly pea.

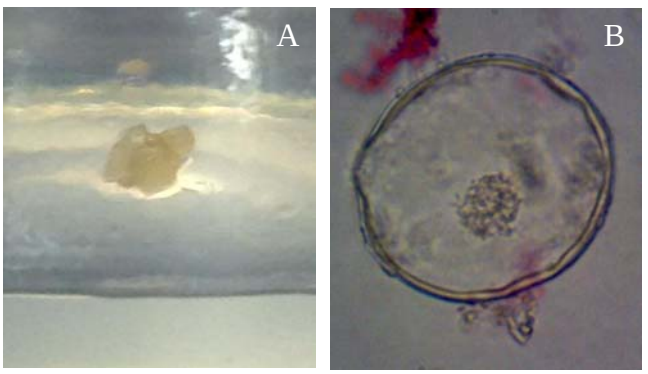

Figure 2. (A) a group of globular embryos formed in excised anther explants from butterfly pea flower of stage 2 development cultured on basal Murashige and Skoog medium supplemented with 3 mg/l 2,4-D and 60 g/l sucrose for 30 days; (B) light microscopic observation showing a microspore at the uninucleate stage

When the androgenic embryos of butterfly pea were transferred onto basal MS medium supplemented with 30 g/l sucrose without addition of any plant growth regulators, the globular embryos (Figure 3A) developed further into heart-stage embryo (Figure 3B). Nevertheless, these embryos did not convert into plantlets. Thus, unlike Sturt's desert pea, both globular and heart-stage embryos of butterfly pea seemed to develop directly from excised anther explants without any intervening callus formation. It would appear that different plant species in the same family might have dissimilar responses to a comparable androgenic embryo induction medium.

In conclusion, anther (or microspore) developmental stage and sucrose concentration affected direct androgenic embryo induction in butterfly pea. Further studies are required to refine the media used for androgenic embryo induction and plantlet conversion.

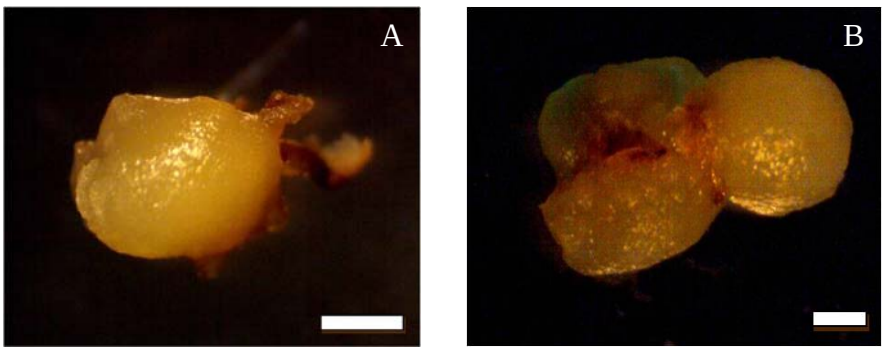

Figure 3. Androgenesis of butterfly pea: (A) globular embryo, (B) heart-stage embryo (scale bar = 1 mm)

References

Armbruster, W. S., Mulder, C. P., Baldwin, B. G., Kalisz, S., Wessa, B. & Nute, H. (2002). Comparative analysis of late floral development and mating-system evolution in tribe Collinsieae (Scrophulariaceae S.L.). *American Journal of Botany*, *89*(1), 37-49. http://dx.doi.org/10.3732/ajb.89.1.37

Barik, D. P., Naik, S. K., Mudgal, A. & Chand, P. K. (2007). Rapid plant regeneration through in vitro axillary shoot proliferation of butterfly pea (*Clitoria ternatea* L.)—a twinning legume. *In Vitro Cellular and Developmental Biology - Plant*, *43*(2), 144-148. http://dx.doi.org/10.1007/s11627-007-9040-y

Forster, B. P., Heberle-Bors, E., Kasha, K. J. & Touraev, A. (2007). The resurgence of haploids in higher plants. *Trends in Plant Science*, *12*(8), 368-375. http://dx.doi.org/10.1016/j.tplants.2007.06.007

Gomez, S. M. & Kalamani, A. (2003). Butterfly pea (*Clitoria ternatea*): a nutritive multipurpose forage legume for the tropics - an overview. *Pakistan Journal of Nutrition*, *2*(6), 374-379. http://dx.doi.org/10.3923/pjn.2003.374.379

Krzyżanowska, D. & Górecka, K. (2008). Effect of various stress factors on the induction of androgenesis in anther cultures of brussels sprouts (*Brassica oleracea* L. var. *gemmifera*). *Vegetable Crops Research Bulletin*, *69*, 5-13. https://doi.org/10.2478/v10032-008-0015-2

Maraschin, S. F., de Priester, W., Spaink, H. P. & Wang, M. (2005). Androgenic switch: an example of plant embryogenesis from the male gametophyte perspective. *Journal of Experimental Botany*, *56*(417), 1711-1726. https://doi.org/10.1093/jxb/eri190

Morris, J. B. (2009). Characterization of butterfly pea (*Clitoria ternatea* L.) accessions for morphology, phenotype, reproduction and potential nutraceutical, pharmaceutical trait utilization. *Genetic Resources and Crop Evolution*, *56*, 421-427.

Mukhtar, S., Ahmad, N., Khan, M. I., Anis, M. & Aref, I. M. (2012). Influencing micropropagation in *Clitoria ternatea* L. through the manipulation of TDZ levels and use of different explant types. *Physiology and Molecular Biology of Plants*, *18*(4), 381-386. http://dx.doi.org/10.1007/s12298-012-0136-4

Murashige, T. & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Plant Physiology*, *15*, 473-497. http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nair, D. S. & Reghunath, B. R. (2008). Effective use of encapsulation-dehydration technique in cryopreserving somatic embryos of butterfly pea (*Clitoria ternatea* L.). *Journal of Herbs, Spices & Medicinal Plants*, *13*(3), 83-95. http://dx.doi.org/10.1300/J044v13n03_07

Osolnik, B., Bohanec, B. & Jelaska, S. (1993). Stimulation of androgenesis in white cabbage (*Brassica oleracea* var. *capitata*) anthers by low temperature and anther dissection. *Plant Cell, Tissue and Organ Culture*, *32*(2), 241-246. http://dx.doi.org/10.1007/BF00029849

Panchangam, S. S., Mallikarjuna, N., Gaur, P. M. & Suravajhala, P. (2014). Androgenesis in chickpea: anther culture and expressed sequence tags derived annotation. *Indian Journal of Experimental Biology*, *52*(2), 181-188.

Sudhersan, C., Jibi Manuel, S. & Al-Sabah, L. (2008). Haploid plant production from pollen grains of Sturt's desert pea via somatic embryogenesis. *American-Eurasian Journal of Scientific Research*, *3*(1), 44-47.

Wang et al. (2010). Microsporogenesis and pollen formation in cassava. *Biologia Plantarum*, *55*(3), 469-478. http://dx.doi.org/10.1007/s10535-011-0112-9

Willcox, M. C., Reed, S. M., Burns, J. A. & Wynne, J. C. (1991). Effect of microspore stage and media on anther culture of peanut (*Arachis hypogaea* L.). *Plant Cell, Tissue and Organ Culture*, *24*(1), 25-28. http://dx.doi.org/10.1007/BF00044261