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Towards Sterile Plant Production in Sturt's Desert Pea (*Swainsona formosa*) via Anther Culture

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Abstract

Sturt's Desert Pea, *Swainsona formosa*, (G.Don) J.Thompson, is a legume native to Australia with a vibrant colour of flowers. The economic importance of this plant is in its ornamental use in hanging baskets and containers or for cut flowers both in Australia and abroad. The production of a large amount of pollen grains in the flower is a major impediment in the commercialisation of this plant. Petal staining by pollen as well as self-pollination during transport reduces the quality of flowers. Producing sterile plants via anther culture is, therefore, the focus of present work. Anthers from floral buds approximately 1.3 – 1.5 cm long were obtained from glasshouse grown plants. After surface sterilisation in 70% ethanol for 10 seconds anthers were dissected out of the buds and their filaments were removed. The anthers were cultured on B5 medium supplemented with vitamins and 2% sucrose. The effect of media types (solid, liquid, paper bridges), light spectra [white (390-760nm); blue (450-550nm); green (492-550nm); yellow (550-588nm); red (647-770nm); and darkness], microspore developmental stages (mother cell – microspores) and plant growth regulators (auxins + cytokinins) were investigated. Androgenesis was not achieved in any of the treatments applied or at any developmental stage tested. Callus was produced on anthers when media were supplemented with plant growth regulators. The type, concentration and combination of plant growth regulators affected colour and texture of calli. The calli ranged from nodular and compact to spongy and friable with a wide range of colours. In subsequent subculturing of calli only those cultured on indole butyric acid and kinetin produced shoots or roots, but these cultures degenerated within 8 weeks of subculture. Work in progress is aimed at determining the effect of anther pre-treatment in haploid plant production, as well as the causes of culture decline in Sturt's Desert Pea.

Keywords: legume, media, light, microspore developmental stage, plant growth regulator, androgenesis, *Clianthus formosus*

INTRODUCTION

Sturt's Desert Pea, *Swainsona formosa* (G.Don) J.Thompson (syn. *Clianthus formosus* (G.Don) Ford & Vick.), is a legume native to Australia. It has flowers with vibrant colours (Williams and Taji, 1991) and is the floral emblem of South Australia (Kirby, 1996a). The economic importance of this species is its potential use as a hanging basket, container or cut flower plant (Williams and Taji, 1991; Kirby, 1996a,b). It is not only in demanded by the domestic cut flower market, but also overseas. A market survey conducted by South Australian Agriculture revealed that Japan is one of

the most important cut flower markets where Sturt's Desert Pea has already been exported (Barth and Bennel, 1989).

However, the commercialisation of Sturt's Desert Pea as cut flowers is hampered by petal staining of pollen grains which are shed during transportation, reducing the flower quality significantly. In addition, during transportation self pollination may take place, reducing the vase life of the flowers. Therefore, developing strategies to produce pollenless plants is the most appropriate method to solve this problem.

Previous attempt to produce haploid Sturt's Desert Pea via androgenesis have been unsuccessful (Tade, 1992). This research represents further investigation of factors which may influence the success of anther culture of this species. The effect of media types (solid, liquid, paper bridges), light spectra [white (390-760nm); blue (450-550nm); green (492-550nm); yellow (550-588nm); red (647-770nm); and darkness], microspore developmental stage (pre-tetrad, tetrad and post-tetrad) and plant growth regulators, as well as problems related to *in vitro* differentiation and regeneration were investigated as the first step in this endeavour.

MATERIALS AND METHODS

Plant materials were grown in a temperate glasshouse at day/night temperature of 30/20ºC and light intensity ranging from 650 to 1200 µmol m^{-2} s^{-1}. Anthers from floral buds of 1.3 – 1.6 mm long (depending on experiment) were isolated and surface sterilised with 70% alcohol for approximately 10 sec. In a laminar air flow cabinet (LAFC), the outer parts of floral buds were removed to expose the anthers. The filament was removed prior to culture on B5 medium (Gamborg *et al.*, 1968) supplemented with 2% sucrose.

Cultures were kept in a culture room at a temperature of 25 ± 1ºC under cool white fluorescent light at an intensity of 50 µmol m^{-2} s^{-1} (depending on experiment) and a 16/8 h photoperiod for 8 weeks. Ten anthers originating from one bud were cultured in one vessel. A completely randomised design was used in this experiment and analysis of variance (ANOVA) was employed in data analyses where applicable.

The Effect of Media

Anthers with microspores at tetrad stage (Figure 1) were cultured onto solid and liquid media, and on a paper bridge made from Whatman No. 1 filter paper. The paper bridge was used only in liquid media. The culture media were supplemented with

vitamins but without plant hormones. Cultures were kept at a temperature of 25 ± 1°C and under a 16/8 h photoperiod using cool white fluorescent lamps for 8 weeks.

A completely randomized experimental design was used with 3 treatments (culture media: solid, liquid and paper bridge) and 10 replicates. Each replicate consisted of 5 florets, and each floret contained 10 anthers. Observation was made on weekly basis and data were recorded at the end of culture period (week 8).

The Effect of Light Spectra

The stage of microspore development and medium used were the same as the previous trial. Culture were kept under various light spectra: blue (455-500 nm), green (500-560 nm), yellow (565-595 nm), red (640-700 nm) and white with light intensity ranging from 9 to 14 µmol m^{-2} s^{-1} (depending on light spectrum), at photoperiods of 16/8 h and a 24-h dark condition treatments. The temperature regime under which the cultures were incubated was similar to the previous trial.

A completely randomized design with 10 replicates was used in this trial. Each replicate consisted of 10 anthers obtained from 1 flower bud. Weekly observations were made and data were recorded at the end of culture period (week 8).

The Effect of Microspore Developmental Stage

Microspore developmental stage trial used anthers of three different developmental stages: microspore mother cell, tetrad to late uninucleate and microspore (Figure 1). Anthers were cultured on solid B5 medium supplemented with vitamins but without plant hormones. Cultures were kept at a temperature of 23 ± 2°C and under a 16/8 h photoperiod using cool white fluorescent lamps for 8 weeks.

The experimental design used was completely randomized design with 3 developmental stages as treatments and 10 replicates. Each replicate consisted of 5 Petri dishes which contained 10 anthers from the same flower bud. Observations were made on weekly basis and data were recorded at the end of culture period (week 8).

The Effect of Plant Growth Regulators

Auxin IAA at concentration of 0.57, 5.71, 57.1 µM and IBA at concentration of 0.49, 4.93, 49.3 µM were used in combination with either BA at concentration of 0.44, 4.44, 44.4 µM or kinetin at concentration of 0.46, 4.63, 46.3 µM or 2iP at concentration of 0.49, 4.93, 49.3 µM or zeatin at concentration of 0.46, 4.57, 45.7 µM. Each

treatment consisted of 5 replicates (i.e. flowers from 5 plants), each with 10 anthers from one floret. A completely randomised design was employed here.

Observation was made on weekly basis and data were recorded at the end of culture period (week 16). Data on percentage of anthers forming callus per experimental unit were analysed using ANOVA and means were compared using Fisher's protected least significant difference (FPLSD) (Fisher, 1966).

RESULTS

The Effect of Media

Anthers appeared to be fresh for the first 2 weeks of culture on all media types. At week 3, anthers cultured on solid medium shriveled and gradually turned brown. Browning was also found on anthers cultured on paper bridge. Anthers cultured in a liquid medium however, did not show any changes in colour and shape. Until week 8 of culture, anthers cultured in liquid medium remained white (Table 1; Figure 2).

The Effect of Light Spectra

Callus formation along with anther shriveling were found on anthers cultured under red, yellow, and in continuous dark condition. Anthers cultured under white and green light produce less callus but became swollen, and green-yellow spots were observed inside the anthers. Meanwhile, no response was observed on anthers cultured under blue light (Table 2; Figure 3).

The Effect of Microspore Developmental Stages

Anther culture utilising floral buds ranged in size from 1.3 – 1.5 cm includes all the microspores developmental stages (mother cell, tetrad – uninucleate, microspores). But unfortunately, no microspore-derived embryos were initiated following the attempted culture. Most anthers were shriveled with or without callus formation, and the colour changed into white, white to brown and brown (Table 3; Figure 4).

The Effect of Plant Growth Regulators

The application of all auxins and cytokinins mostly resulted in only callus formation on the anther surface. A number of anthers cultured in some auxins + cytokinins at certain concentrations, as well as the control treatment did not show any response up to 4 months of culture.

In addition, there were also variations in the properties of callus depending on the type of plant hormones being employed. The structure of callus was nodular and compact to spongy and friable, and the colour was white to green. In subsequent subculturing of calli only those cultured on indole butyric acid and kinetin, produced shoots or roots but these cultures degenerated within 8 weeks of subculture.

The effect of auxins and cytokinins sources on the growth of SDP anther cultured *in vitro* is presented in the following sections.

1. **The Effect of IAA + BA.** IAA + BA significantly affected callus formation on *S. formosa* anthers cultured *in vitro* ($P < 0.05$). After 16 weeks in culture, IAA at 5.71 µM + BA at 4.4 µM, and IAA at 57.1 µM + BA at 44.4 µM produced callus at a frequency of 26% (Table 4; Figure 5.A). Increased levels of BA at all IAA levels had higher frequency of callus formation.
2. **The effect of IAA + zeatin.** No significant response was detected on *S. formosa* anthers cultured on medium supplemented with varying concentrations of IAA + zeatin ($P > 0.05$) (Figure 5B).
3. **The effect of IBA + BA.** Anthers of *S. formosa* cultured *in vitro* responded significantly to IBA + BA ($P < 0.05$). The highest number of callus formation (38%) was obtained with the application of IBA at 49.3 µM + BA at 0.44 µM after 16 weeks of culture (Table 5; Figure 5.C).
4. **The effect of IBA + 2iP.** The use of IBA + 2iP significantly affected callus formation on anther culture of *S. formosa* ($P < 0.05$). After 16 weeks in culture IBA 44.4 µM + 2iP 4.93 µM produced callus on 36% of cultured anthers (Table 6; Figure 5.D).
5. **The effect of IBA + kinetin.** Significant response in the form of callus formation was also observed on anther of *S. formosa* cultured on IBA + kinetin ($P < 0.05$). The highest rate of callus formation (36%) was obtained after 16 weeks in culture with the use of IBA 4.93 µM + kinetin 4.63 µM (Table 7; Figure 5.E).
6. **The effect of IBA + zeatin.** IBA + zeatin significantly affected callus formation on anther culture of *S. formosa* ($P < 0.05$). After 16 week in culture, IBA 0.49 µM + zeatin 4.61 µM produced 36% callus formation (Table 8; Figure 5.F).

DISCUSSION

The induction of androgenesis, i.e. plant regeneration from microspores cultured *in vitro* has been known to be affected by a number of factors. Some of these factors

including media type, light spectra, microspore developmental stages and plant hormones were evaluated in present experiments on anther culture of *Swainsona formosa*. However, none of them were found to be effective in inducing plant regeneration from microspores.

Various media types have been used in tissue culture systems, depends on the objectives of the work and explants used. Most anther culture systems are carried out on media solidified with various concentration of agar (Bishnoi *et al.*, 2000). However, higher rate of success for certain species or cultivars have been obtained using liquid media (Trottier *et al.*, 1993; Cistué *et al.*, 1998; Castillo *et al.*, 2000).

Liquid medium containing Ficoll™, a nonionic copolymer of sucrose and epichlorohydrin, was more efficient than solid medium, gelatinous medium or liquid medium and a membrane raft (Hoechst Celanese Corp.) in wheat anther culture (Trottier *et al.*, 1993). The addition of Ficoll™ to liquid medium increased the number of embryos and green plants in anther culture of barley (Cistué *et al.*, 1998) due to its effect on the viscosity of the medium. Inclusion Ficoll™ in Sturt's Desert pea anther culture may bear positive results.

Another important factor that should be taken into account regarding media for anther culture is the nutrient requirement. The composition of B5 medium (Gamborg *et al.*, 1968) was found to be useful in root culture of soybean (Leguminosae). Other nutrient compositions such as that in MS (Murashige and Skoog, 1962) was broadly used in anther culture of various dicots, N6 (Chu *et al.*, 1975) in rice, NLN (Lichter, 1982) in *Brassica napus*, and W14 (Ouyang *et al.*, 1988) and HNG (Chu *et al.*, 1990) in wheat anther cultures. Since the results of present work were obtained from culture utilising only one medium composition (B5), future work in anther culture of Sturt's Desert Pea should investigate effects of different media composition.

In addition to media, growth and development of cultured tissue is also known to be affected by light. Red and blue regime were proven to be important for most plant species, but the present study revealed that no response was obtained on *S. formosa* anthers cultured under blue light spectrum. Blue and white light spectra were recorded to be important in *in vitro* culture of *Melaleuca alternifolia* (Johnson *et al.*, 1996). However, little was known regarding the effect of light spectra on androgenesis.

Microspore developmental stage has been known to be a critical factor in androgenesis in most plant species. The late uninucleate stage was proven to be useful in androgenesis of *Nicotiana tabacum* (Sunderland, 1974), linseed (*Linum*

usitatissimum) (Nichterlein and Friedt, 1993) and *Phleum pratense (Guo et al., 1999)*. Although the late uninucleate to early binucleate stages appear to be an optimum stage of microspore development for most species (Palmer and Keller, 1997), the exact stage for successful androgenesis is species dependent. Furthermore, it is important to note that the conversion rate of microspores to plantlets is very low ranging from 0.2 to 0.5 in wheat (Masojc *et al.*, 1993), 30.6 in rye (Immonen and Anttila, 1999) and 0.5 to 17.6 in triticale (Immonen and Robinson, 2000). The reason for this low conversion rate depends on the exact timing of mitotic division in microspores used. Conversion used only in those microspores that, at the time of introduction to culture, are actively dividing.

Plant growth regulators play a very important role in androgenesis. Most tissue culture system, including anther culture, incorporated various sources of auxins and cytokinins at various concentrations. The response however, differed widely among plant species (Marinkovic and Radojevic, 1992; Baldursson *et al.*, 1993; Nichterlein and Friedt, 1993; Zhong *et al.*, 1995). The mechanism by which plant growth regulators, particularly auxins and cytokinins, influence the growth and development of cultured tissues have been explored by many authors. In our study, the response of *S. formosa* anther to various types and concentrations of auxin and cytokinin was unspecific.

CONCLUSION

No plantlet regeneration was achieved during the experiment. This indicated that there are other factors affecting androgenesis in *S. formosa* in addition to media types, light spectra, microspore developmental stages and plant growth regulators. For success of androgenesis, some other factors need to be optimised including genotype of donor plant and its growth conditions, culture temperature, nutrient requirements and pre-treatment of anthers before culture initiation.

REFERENCES

Baldursson S., Krogstrup P., Nørgaard J.V., and Andersen S.B. (1993). Microspore embryogenesis in anther culture of three species of *Populus* and regeneration of dihaploid plants of *Populus trichocarpa*. *Canadian Journal for Forestry Research* **23**, 1821-1825.

Barth G.E., and Bennel M. (1989). "Market Development of Sturt's Desert Pea (*Clianthus formosus*) for The Cut Flower Market in Japan." South Australia Department of Agriculture, Adelaide.

Bishnoi U., Jain R.K., Rohilla J.S., Chowdhury V.K., Gupta K.R., and Chowdury J.B. (2000). Anther culture of recalcitrant *indica* x Basmati rice hybrids. *Euphytica* **114**, 93-101.

Castillo A.M., Vallés M.P., and Cistué L. (2000). Comparison of anther and isolated microspore cultures in barley. Effects of culture density and regeneration medium. *Euphytica* **113**, 1-8.

Chu C.C., Hill R.D., and Brulé-Babel A.L. (1990). High frequency of pollen embryoid formation and plant regeneration in *Triticum aestivum* L. *Plant Science* **66**, 255-262.

Chu C.C., Wang C.C., Sun C.S., Hsu C., Yin K.C., Chu C.Y., and Bi F.Y. (1975). Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. *Scientia Sinica* **18**, 659-668.

Cistué L., Ramos A., and Castillo A.M. (1998). Influence of anther pretreatment and culture medium composition on the production of barley doubled haploids from model and low responding cultivars. *Plant Cell, Tissue and Organ Culture* **55**, 159-166.

Fisher R.A. (1966). "The Design of Experiment," 8/Ed. Hafner, New York.

Gamborg O.L., Millers R.A., and Ojima K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* **50**, 151-158.

Guo Y.-D., Sewón P., and Pulli S. (1999). Improved embryogenesis from anther culture and plant regeneration in timothy. *Plant Cell, Tissue and Organ Culture* **57**, 85-93.

Immonen S., and Anttila H. (1999). Cold pretreatment to enhance green plant regeneration from rye anther culture. *Plant Cell, Tissue and Organ Culture* **57**, 121-127.

Immonen S., and Robinson J. (2000). Stress treatment and ficoll for improving green plant regeneration in triticale anther culture. *Plant Science* **150**, 77-84.

Johnson K.A., Swiderski A., Bach A., and Pawlowska B. (1996). Effect of light quality on growth and development of *Melaleuca alternifolia* (Myrtaceae) *in vitro*. *In* "Vth International Association for Plant Tissue Culture (Australian Branch) Conference" (A. Taji and R. Williams, eds.), pp. 47-52. University of New England, Armidale, NSW, Australia.

Kirby G.C. (1996a). Sturt's Desert Pea as cut flower crop. *In* "4th National Workshop for Australian Flower", pp. 204-209, Perth, Australia.

Kirby G.C. (1996b). Sturt's Desert Pea for pot plant and hanging baskets. *In* "4th National Workshop for Australian Flower", pp. 44-48, Perth, Australia.

Lichter R. (1982). Induction of haploid plants from isolated pollen of *Brassica napus*. *Zeitschrift für Pflanzenphysiologie* **105**, 427-434.

Marinkovic N., and Radojevic L. (1992). The influence of bud length, age of the tree and culture media on androgenesis induction in *Aesculus carnea* Hayne anther culture. *Plant Cell, Tissue and Organ Culture* **31**, 51-59.

Masojc P., Lukow O.M., McKenzie R.I.H., and Howes N.K. (1993). Responsiveness to anther culture in cultivars and F_1 crosses of spring wheat. *Canadian Journal of Plant Science* **73**, 777-783.

Murashige T., and Skoog F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497.

Nichterlein K., and Friedt W. (1993). Plant regeneration from isolated microspores of linseed (*Linum usitatissimum* L.). *Plant Cell Reports* **12**, 426-430.

Ouyang J.W., Jia S.G., Zhang C., Chen X.D., and Feng G.H. (1988). "A new synthetic medium (W14 medium) for wheat anther culture." Institute of Genetics, Academica Sinica, Beijing.

Palmer C.E., and Keller W.A. (1997). Pollen Embryos. *In* "Pollen Biotechnology for Crop Production and Improvement" (K. R. Shivana and V. K. Sawhney, eds.), pp. 392-422. Cambridge University Press, Cambridge.

Sunderland N. (1974). Anther Culture as a Means of Haploid Induction. *In* "Proceedings of the First International Symposium", pp. 91-122. University of Guelph, Guelph.

Tade E. (1992). Anther and ovule culture of *Clianthus formosus*. Master of Rural Science, University of New England, Armidale.

Trottier M.-C., Collin J., and Comeau A. (1993). Comparison of media for their aptitude in wheat anther culture. *Plant Cell, Tissue and Organ Culture* **35**, 59-67.

Williams R., and Taji A. (1991). Sturt's Desert Pea in review. *In* "Australian Horticulture", Vol. 89, pp. 85-88.

Zhong D., Michaux-Farriére N., and Coumans M. (1995). Assay for doubled haploid sunflower (*Helianthus annuus*) plant production by androgenesis: fact or artifact? Part 1. *In vitro* anther culture. *Plant Cell, Tissue and Organ Culture* **41**, 91-97.

APPENDIX

Table 1. The appearance of *S. formosa* anthers cultured on 3 different types of media.

Media type	No of replicates	No. of anthers plated	Anthers' performance
Solid	10	500	brown + shriveled + callus
Liquid	10	500	white
Paper bridge	10	500	brown

Table 2. The appearance of *S. formosa* anthers cultured under 6 different light spectra

Light spectra	No of replicates	No. of anthers plated	Type of responses
White	10	500	Swollen and yellow spots inside anthers
Red	10	500	Callus formation and shriveled
Yellow	10	500	Callus formation and shriveled
Green	10	500	Swollen and green spots inside anthers
Blue	10	500	-
Dark	10	500	Mainly callus formation

Table 3. The effect of developmental stage on anther culture of *S. formosa*

Developmental stage	Reps.	No. of anthers plated	Nature of response		
			Callus (%)	Shriveled (%)	colour
Mother cell	1	50	-	6	white to brown
	2	50	4	12	white
	3	50	2	8	white to brown
	4	50	-	10	white to brown
	5	50	6	8	brown
	6	50	-	6	brown
	7	50	10	2	brown
	8	50	-	10	white to brown
	9	50	-	4	white
	10	50	2	2	white
Tetrad - late uninucleate	1	50	-	6	white
	2	50	-	10	white
	3	50	6	6	white
	4	50	10	14	white to brown
	5	50	12	16	white to brown
	6	50	4	6	white to brown
	7	50	-	4	white
	8	50	8	2	white
	9	50	4	2	white to brown
	10	50	-	2	white
Microspore	1	50	6	18	brown
	2	50	-	10	white to brown
	3	50	12	6	white to brown
	4	50	-	10	brown
	5	50	-	18	brown
	6	50	2	8	white to brown
	7	50	-	8	white to brown
	8	50	2	4	white to brown
	9	50	-	4	white to brown
	10	50	8	2	white

Table 4. The effect of IAA + BA on callus formation in anther culture of *S. formosa*.

Hormone concentration (µM)		Number of anthers plated	Mean number of callus (%)
IAA	BA		
0.57	0.44	50	16 a
0.57	0.44	50	16 a
0.57	0.44	50	18 ab
5.71	4.44	50	22 bc
5.71	4.44	50	24 c
5.71	4.44	50	26 c
57.1	44.4	50	18 ab
57.1	44.4	50	24 c
57.1	44.4	50	26 c

Five replicates were maintained for each treatment. Control was excluded from analysis because the response was nil. Mean values with the same letter are not significant ($FPLSD_{0.05}$ = 4.67)

Table 5. The effect of IBA + BA on callus formation in anther culture of *S. formosa*.

Hormone concentration (µM)		Number of	Mean number of
IBA	BA	anthers plated	callus (%)
0.49	0.44	50	20 a
0.49	4.44	50	32 b
4.93	0.44	50	22 a
4.93	4.44	50	24 a
49.3	0.44	50	38 b
49.3	4.44	50	24 a

Five replicates were maintained for each treatment. Control, IBA 0.49 µM + BA 44.4 µM, IBA 4.93 µM + BA 44.4 µM and IBA 49.3 µM + BA 44.4 µM were excluded from analysis because the response was nil. Mean values with the same letter are not significant ($FPLSD_{0.05}$ = 4.13)

Table 6. The effect of IBA + 2iP on callus formation in anther culture of *S. formosa*.

Hormone concentration (µM)		Number of	Mean number of
IBA	2iP	anthers plated	callus (%)
0.49	0.49	50	24 a
0.49	4.93	50	24 a
4.93	0.49	50	24 a
4.93	4.93	50	32 b
49.3	0.49	50	24 a
49.3	4.93	50	36 b

Five replicates were maintained for each treatment. Control, IBA 0.49 µM + 2iP 49.3 µM, IBA 4.93 µM + BA 49.3 µM and IBA 49.3 µM + BA 49.3 µM were excluded from analysis because the response was nil. Mean values with the same letter are not significant ($FPLSD_{0.05}$ = 4.91)

Table 7. The effect of IAA + kinetin on callus formation in anther culture of *S. formosa*.

Hormone concentration (µM)		Number of	Mean number of
IAA	kinetin	anthers plated	callus (%)
0.49	0.46	50	28 a
0.49	0.46	50	34 b
0.49	0.46	50	28 a
4.93	4.63	50	28 a
4.93	4.63	50	36 b
4.93	4.63	50	34 b
49.3	46.3	50	28 a
49.3	46.3	50	28 a
49.3	46.3	50	32 ab

Five replicates were maintained for each treatment. Control was excluded from analysis because the response was nil. Mean values with the same letter are not significant ($FPLSD_{0.05}$ = 4.37)

Table 8. The effect of IBA + 2iP on callus formation in anther culture of *S. formosa*.

Hormone concentration (µM)		Number of	Mean number of
IBA	zeatin	anthers plated	callus (%)
0.49	4.61	50	36 b
0.49	46.1	50	26 a
4.93	4.61	50	32 b
4.93	46.1	50	26 a
49.3	4.61	50	34 b
49.3	46.1	50	26 a

Five replicates were maintained for each treatment. Control, IBA 0.49 µM + zeatin 0.46 µM, IBA 4.93 µM + zeatin 0.46 µM and IBA 4.93 µM + zeatin 0.46 µM were excluded from analysis because the response was nil. Mean values with the same letter are not significant ($FPLSD_{0.05}$ = 4.91)

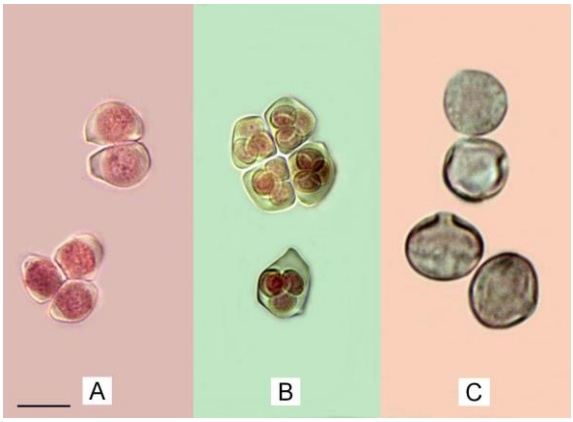

Figure 1. Microspores at 3 different developmental stages used as planting materials. A, pollen mother cell tetrad; B, tetrad; C, pollen grain. Bar (A, B) = 25 µm, C = 10 µm.

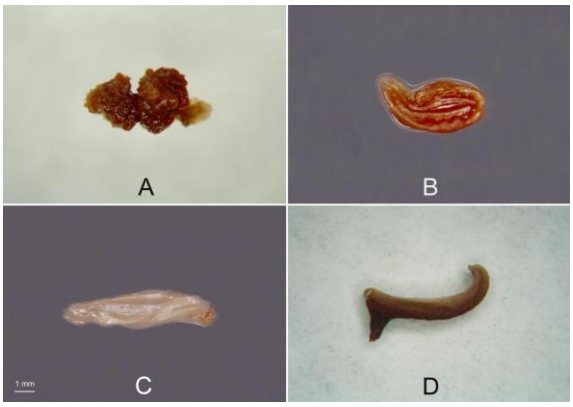

Figure 2. The response of *S. formosa* anthers to various media. A, brown callus produced on solid medium; B, brown and shriveled anther on solid medium; C, white anther in liquid medium; D, brown anther on paper bridge.

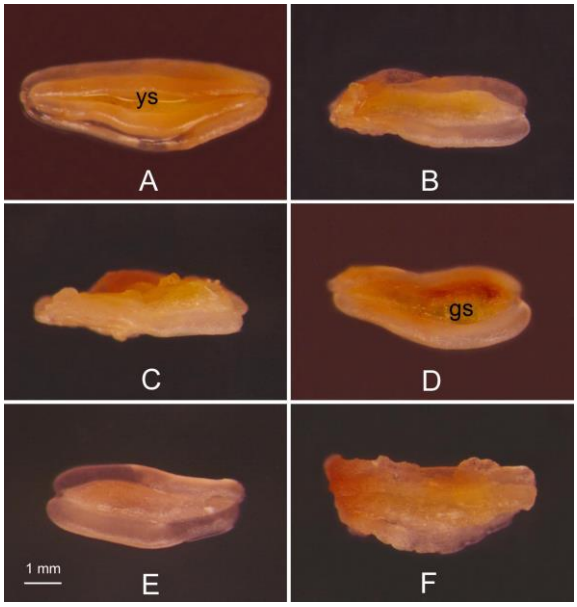

Figure 3. Responses of *S. formosa* anthers cultured under different light spectra. A, swollen anther with yellow spots (ys) under white light; B, callus formed under red light; C, callus formed under yellow light; D, swollen with green spots (gs) under green light; E, no response under blue light; F, callus formed under continuous dark condition.

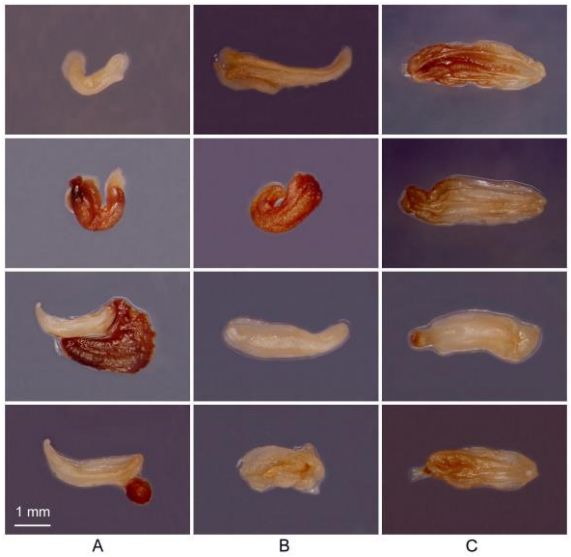

Figure 4. The nature of *S. formosa* anthers from three different developmental stage (8 weeks after culture initiation). A, anthers containing microspores at mother cell stage; B, anthers containing microspores at tetrad – late uninucleate stage; C, anthers containing microspores at pollen grain stage.

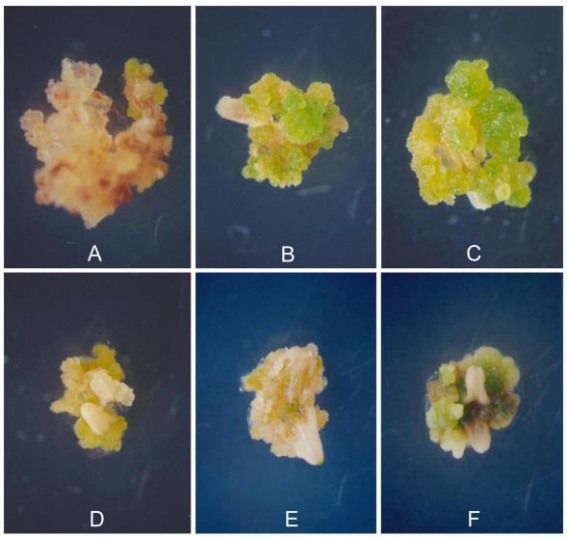

Figure 5. Some examples of callus formed under different combinations of auxins + cytokinins. A, IAA 57.1 µM + BA 44.4 µM; B, IAA 57.1 µM + zeatin 46.1 µM; C, IBA 49.3 µM + BA 0.44 µM; D, IBA 49.3 µM + 2iP 4.93 µM; E, IBA 49.3 µM + kinetin 4.63 µM; F, IBA 49.3 µM + zeatin 4.61 µM.