

SHORT COMMUNICATION

Pretreatment Stress Enhances Embryogenic Callus Production in Anther Culture of Sturt's Desert Pea (*Swainsona formosa*)

ZULKARNAIN

Faculty of Agriculture, University of Jambi, Kampus Pinang Masak, Mendalo, Jambi 36361, Indonesia
Phone/Fax. +62-741-582781, E-mail: dr.zulkarnain@yahoo.com

Received January 31, 2005/Accepted January 24, 2007

The aims of the experiment were to investigate the effect of pretreatment stress on the proliferation of anther-derived embryogenic callus, and its subsequent embryo regeneration in *Swainsona formosa*. Anthers were pretreated in starvation medium at cold (4 °C) temperature and cultured. Cultures were initially placed in darkness for four weeks, followed by 16-h photoperiod at 25 ± 1 °C. The stress pretreatment was found to enhance embryogenic callus formation on cultured anthers. Further, somatic embryos were successfully regenerated from embryogenic callus upon transferred onto regeneration medium supplemented with 1% sucrose and 4.63 µM kinetin, and later plantlets were obtained. Meanwhile, nonembryogenic callus continued to grow resulting in more callus mass without showing any embryoid initiation.

Key words: anther culture, embryogenic callus, stress pretreatment, *Swainsona formosa*

Sturt's desert pea (*Swainsona formosa* (G. Don) J. Thompson), known to aborigines as Marlukuru, is one of Australia's most spectacular wild flowers, and is the floral emblem of South Australia. Its large flag-shaped flowers, colored bright red (or pure white to deep purple in some wild populations), have made this plant one of most striking flowering plants in the world (Taji 1997).

Natural propagation of this species is through seeds, which requires scarification in boiling water for about ten seconds to assist germination. Clonal propagation using cutting results in prostrate creeping plants that are undesirable for pot or cut flower production, due to poor root system. A micropropagation system has been developed by Williams and Taji (1987) and Sudharsan and Abo El-Nil (2002) using stem segments, petiole and leaflets as starting materials. However, the recalcitrance of legume tissues caused difficulties in regenerating plants under *in vitro* system (Van Doorne *et al.* 1995). For this reason, Lakshmanan and Taji (2000) suggested that different environmental stresses might be useful to induce somatic embryogenesis in such recalcitrant tissues. These stress pretreatments of anthers are of critical importance for blocking gametophytic development and for triggering pollen embryogenesis in competent microspores (Touraev *et al.* 1997). We reported here the enhancement of callus proliferation following anther stress pretreatment, and somatic embryo production from anther-derived callus upon cultured on a double-phase medium.

Anthers of *S. formosa* were pretreated with water or starvation medium at cold (4 °C) or room temperature (25 °C) for 2, 4, 6, or 8 days before culture initiation. The starvation medium contained 1.49 g l⁻¹ KCl, 0.12 g l⁻¹ MgSO₄, 0.11 g l⁻¹ CaCl₂, 0.14 g l⁻¹ KH₂PO₄, and 54.7 g l⁻¹ mannitol at pH 7.0 (Kyo

& Harada 1986). The basal medium used was B5 (Gamborg *et al.* 1968) supplemented with vitamins, 2% sucrose, 49.3 µM IBA, 4.61 µM zeatin, and solidified with 0.5% Bitek™ agar.

Anthers were plated horizontally on the surface of agar medium, and one drop of Ficoll-400® at concentration of 10% (w/v) was added on cultured anthers to perform a double-phase medium. Cultures were incubated at 25 ± 1 °C in darkness for 4 weeks, then transferred in 16-h photoperiod at light intensity at 50 µM m⁻² s⁻¹ for the rest of culture period to allow callus to grow further.

Ten replicates, each consisted of five plates containing ten anthers within each plate, were used in a complete randomized design. Data on the percentage of explants forming callus were recorded, and standard deviation of treatment mean was calculated.

Callus started to proliferate within two weeks of culture, and continued to grow until for another two weeks in darkness. All callus had similar properties, i.e. they were compact, and white translucent with glossy appearance. No embryo formation was found at this stage. Following four weeks of transfer in light condition, more callus proliferation was observed on cultured anthers, particularly those pretreated with starvation medium at 4 °C (Table 1). These callus were friable, white translucent, and showing nodulations (Figure 1a), and suspected as embryogenic callus. Meanwhile, callus from anther pretreated with water starvation were compact, smooth, and colouring white, showed no nodulations (Figure 1b), and suspected as nonembryogenic callus.

Proliferated callus were subcultured onto regeneration medium with the same composition as the callus induction stage, but supplemented with 1% sucrose and 4.63 µM kinetin.

Table 1. The effect of starvation medium and temperature at different duration of culture on percentage of callus formation on anthers of *S. formosa*

Pretreatment		Duration of culture pretreatment (days)			
Medium	Temperature (°C)	2	4	6	8
Water	4	93.0 ± 5.53*	91.8 ± 4.14	90.4 ± 3.56	90.0 ± 6.26
	25	85.6 ± 6.85	85.6 ± 5.48	85.6 ± 6.24	83.4 ± 5.42
Mannitol	4	98.8 ± 1.33	98.4 ± 1.50	98.4 ± 1.96	98.6 ± 1.28
	25	92.6 ± 2.12	89.8 ± 1.48	86.2 ± 2.90	82.6 ± 2.84

*Values represent the means ± standard deviation based on ten replicates (five plates per replicate and ten anthers per plate)

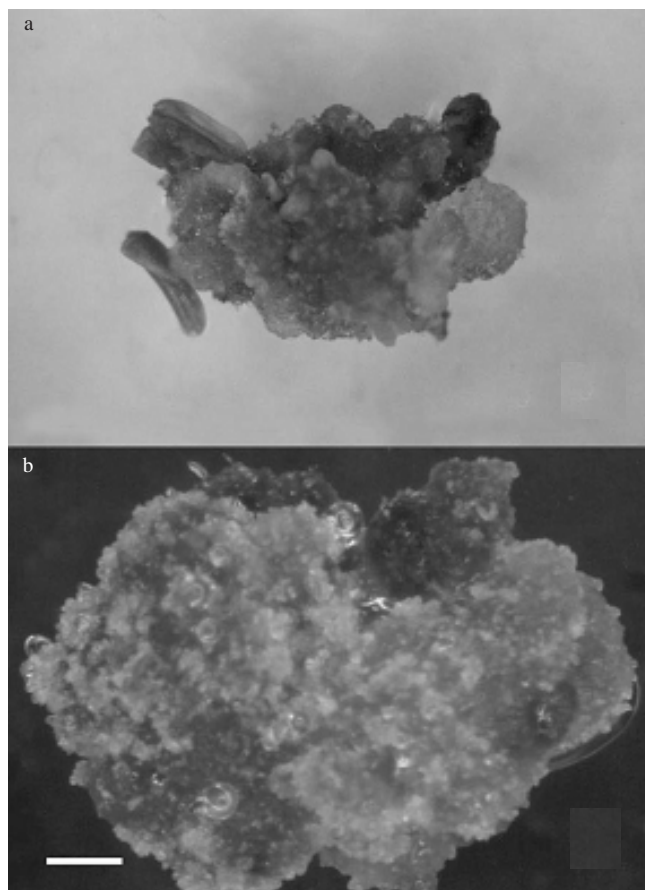


Figure 1. Callus types on anther culture of *S. formosa*. a. embryogenic callus proliferating from anther pretreated with mannitol starvation at 4 °C for 2 days; b. nonembryogenic callus proliferating from anther pretreated with water starvation at 25 °C for 2 days (both cultures are 4 weeks old). Bar for a and b = 0.5 mm.

The double-phase medium with the addition of 10% (w/v) Ficoll-400® was also used. Cultures were placed at 25 ± 1 °C with light intensity at $50 \mu\text{M m}^{-2} \text{s}^{-1}$ and 16-h photoperiod. The growth of cultures was observed for another four weeks to observe the regeneration of somatic embryos on the two groups of callus mass.

Visual observation for embryogenic potential was made for another four weeks of culture under light condition. The embryogenic potential of the callus was determined using light microscope. Callus were grouped into (i) friable, light green with nodulation; and (ii) compact, smooth, coloring white, yellowish or dark green.

Following subculture on new fresh medium callus from group-1 showed their embryogenic potential by regenerating somatic embryos (Figure 2a). The regeneration of somatic

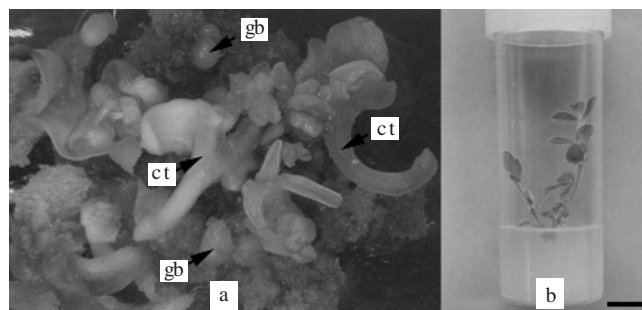


Figure 2. Somatic embryos and plantlet regenerated from anther culture of *S. formosa*. a. somatic embryos at globular (gb) and cotyledon (ct) stages formed from embryogenic callus mass at four weeks after subculture onto a double-phase medium supplemented with $4.62 \mu\text{M}$ kinetin; b. plantlet raised from a somatic embryo at four weeks after embryo formation. Bars: a = 0.5 mm, b = 1.0 cm.

embryos confirmed that the embryogenic potential of *S. formosa* callus was characterized by light green in color as demonstrated by Sudhersan and Abo El-Nil (2002) on *in vitro* culture of this species using stem, petiole and leaflet explants. These somatic embryos developed into plantlets when isolated and cultured on agar solidified B5 medium supplemented with similar level of sucrose and growth regulator (Figure 2b). Other callus mass of group-2 continued to proliferate and produce more callus mass and never produce embryos on the regeneration medium. This was similar to the findings of Tapingkae (1998), that reported no somatic embryos were regenerated from *S. formosa* white callus.

A low temperature pretreatment of floral buds or isolated anthers may enhance embryogenesis, even if donor plants are not initially grown at low temperatures (Palmer & Keller 1997). A significant increase in green plant regeneration is achieved in anther culture of spring and winter rye cultivars when anthers are subjected to a cold (4 °C) pretreatment for 2-4 weeks before being cultured (Immonen & Anttila 1999). Anthers cold pretreatment at 4 °C was found to be useful in *Secale cereale* and triticale anther culture (Immonen & Anttila 1999) and triticale (\times *Triticosecale*) (Immonen & Robinson 2000). In *Hordeum vulgare*, however, better results were obtained with mannitol starvation instead of low temperature pretreatment (Cistué *et al.* 1998). Pretreatment of anthers in a starvation medium supplemented with mannitol was also found to be effective for *Triticum aestivum* androgenesis (Indrianto *et al.* 1999).

The stress pretreatment of anther is of critical importance for blocking gametophytic development and for triggering pollen embryogenesis in competent microspores.

Touraev *et al.* (1997) suggested that the importance of cold pretreatment was attributed to the effect of temperature on tissue metabolism. At low temperature the rate of metabolism slows down due to decrease of enzyme activities. Hence, the gametophytic differentiation is inhibited, and allows cell division and regeneration to occur when the tissue is released from cold pretreatment and subjected to normal *in vitro* condition (Kyo & Harada 1986; Touraev *et al.* 1996; Höfer *et al.* 1999).

Although microspore embryogenesis was not fully accomplished, this study had demonstrated the key role of mannitol starvation coupled with low temperature pretreatment in directing the growth of anther tissue into embryogenic callus and subsequent somatic embryogenesis from anther-derived callus. In tissue culture of recalcitrant plants such as legume species (Taji *et al.* 2002), it would be necessary to combine stress pretreatment with other growth factors (Immonen & Robinson 2000) to effectively induce microspore embryogenesis. Therefore, future work on anther culture of *S. formosa* needs to focus on investigating the effect of mannitol starvation at 4 °C in combination with various cultural conditions.

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