South African Journal of Botany 2001, 67: 358–361
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SOUTH AFRICAN JOURNAL OF BOTANY
ISSN 0254-6299

Short Communication

Establishment of *in vitro* cultures of tree peonies

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Received 5 March 2000, accepted in revised form 5 June 2000

Decontamination of elite dwarf cultivars of the tree peony was increased by immersion of explants (leaves, buds, roots, seeds) in a mixture of 20% ethanol and 0.075% $HgCl_2$ for 7min. Seeds were best decoated and the embryos then disinfested. The phytohormones 2,4-D, BA and GA_3 induced callus on both the embryonic axis and cotyledons. To form callus on young leaves and petioles of the dwarf cultivars 'Xue Li Zi Yu' and 'Zi Xia Lin' 4.52µM 2.4-D and 4.44µM BA were required. Addition of 1.44µM GA_3 to this medium was needed for cv. 'Cai Lan' to survive and ultimately form callus.

Abbreviations: AC = activated charcoal; BA = benzyladenine; cv. = cultivar; GA = gibberellin; GA_1 = gibberellic acid; MS = Murashige and Skoog medium; NAA = α-naphthaleneacetic acid; 2,4-D = 2,4-dichlorophenoxyacetic acid; WPM = Woody Plant Medium

Chinese tree peony *Paeonia suffruticosa* Andr. comprises more than 500 cultivars. These attractive ornamentals have large, colourful flowers sought after in many countries. To maintain desirable hereditary traits the plants are propagated vegetatively. This process is time consuming and slow, contributing to the rarity and high cost of the plants (Aoki and Inoue 1992). As a result, many attempts have been made to micropropagate the plants, using different explants with varying success. Explants used include zygotic embryos (Demois and Partanen 1969), flower buds (Meyer 1976), stems (Gildow and Mitchell 1977) and roots (Meyer 1976, Gildow and Mitchell 1977). Only callus formation was achieved. Plantlet regeneration was achieved from axillary buds, leaves and petioles of cv. 'Qing Long Wo Mo Chi' and #18 (Li *et al.* 1984), stems of cv. 'Papaveracea' (Harris and Mantell 1991), and buds of cv. 'Mme de Vatry' (Bouza *et al.* 1994). Little or no information is available on the elite dwarf cultivars and cv. 'Fengdanbai' which is a hardy plant.

In the case of herbaceous peonies *P. anomala* (Brukhin and Batygina 1994), *P. albiflora* (Kim and Lee 1995), *P. mlokosewitschii* and *P. tenuifolia* (Orilikowska *et al.* 1998) somatic embryogenesis has been achieved. There is no information with respect to *P. suffruticosa*. Before success with this technique can be achieved it is necessary to improve decontamination and minimise excessive culture browning. In addition, it is necessary to obtain an optimal medium containing the most efficient hormone supplements.

Stock plants (5-years-old) of *Paeonia suffruticosa* Andr. cv. 'Cai Lan', 'Xue Li Zi Yu' and 'Zi Xia Lin' were raised in a greenhouse. Upon sprouting, axillary buds, young expanding leaves, fully expanded leaves and active roots (3mm diameter) were used as explants. Seeds of cv. 'Fengdanbai' were collected from field grown plants after the follicles had ripened in autumn. The moisture content of the seed lot was 12% and the mass of 1 000 seeds was 238g. The seeds were stored at 5°C prior to use.

Leaves were first rinsed in running tap water for 20min and then decontaminated in groups using three methods: 1.) Immersion into 70% (v/v) ethanol for 7–10sec and then into 0.5, 1.0 and 3.5% NaOCl (active ingredient in commercial bleach) for 15, 20 and 25min respectively; 2.) Immersion into 70% (v/v) ethanol for 7–10sec and then into 0.1% (w/v) $HgCl_2$ for 3, 5 and 7min respectively; 3.) Immersion in a mixture of 20% (v/v) ethanol and 0.075% (w/v) $HgCl_2$ for 5, 7 and 9min respectively. All explants were subsequently rinsed 3 times (3min each) in sterile distilled water. Tween 20 (1 drop per 50ml) was added to each sterilant. In the case of buds, scales were removed whereafter they were treated as the leaves. Roots were heated in water at 42°C for 2h (Langens-Gerrits *et al.* 1998) prior to decontamination. The procedures used were the same as for the leaves.

Seeds were decontaminated using three methods: 1.) Immersion in 70% (v/v) ethanol for 2min followed by 30min in 3.5% (active ingredient) NaOCl or 10min in 0.1% (w/v) $HgCl_2$ 2.) The seed coat was removed and the embryos were immersed in a mixture of 20% (v/v) ethanol and

0.075% (w/v) $HgCl_2$ for 7min; 3.) A combination of methods 1 and 2. Contaminated seeds were removed after the intact seeds had been in culture for 60 days, the embryos excised and then decontaminated using method 2. The experiments were repeated twice with 25 replicates per treatment and the data subjected to statistical analysis (Steel *et al.*1997).

Three methods were used to reduce browning: 1.) Depending on explant vigour, young (emerged but unexpanded), expanding, fully expanded leaves, and the bud tips (less than 3mm in length) were excised under aseptic conditions and used; 2.) Leaves and buds were soaked in 0.8mM ascorbic acid or 0.8mM citric acid (George and Sherrington 1984) or a combination of these acids (0.4mM each) for 20min prior to decontamination. Explants were then innoculated onto a medium containing 0.4mM of both chemicals; 3.) Leaves and buds were incubated on medium free of activated charcoal (AC) or containing 0.3% (w/v) AC respectively. Browning was recorded every 5 days for 35 days. Each treatment contained 25 replicates. For buds only 5 replicates were available.

Two media, Murashige and Skoog (1962) (MS) and the Woody Plant Medium (WPM) of Lloyd and McCown (1980), previously used for tree peonies, were employed. The pH of these were adjusted to 5.8 and they were solidified with Difco agar (0.8w/v). On WPM signs of a Ca-deficiency were observed. This was rectified by raising the $CaCl_2$ level of the medium to 6mM.

Excised embryos of cv. 'Fengdanbai' were placed on WPM containing 2,4-D (4.52µM), BA (4.44µM), or GA_3 (1.44-2.89µM) respectively. Data were collected every 5 days from 10 replicates. Callus formation, cotyledon expansion and embryo axis elongation were recorded. Mature excised embryos were also cultured on WPM containing 6mM $CaCl_2$, 500mgl^{-1} casein hydrolysate, 1mM glutamate and combinations of 2,4-D (2.26–4.52µM) and BA (2.22–4.44µM). Mass change over time was recorded for 60 days from 20 replicates. For the dwarf cultivars a series of hormone combinations (2,4-D, NAA, BA and GA_3) were used with the standard WPM. Cultures were maintained at 25±2°C under a 16h/8h light/dark regime. Light (23µmol $m^{-2}s^{-1}$) was provided by cool white fluorescent tubes supplied by Phillips (TLD 36W/840).

The disinfestation solution, explant and time applied all influenced the degree of decontamination recorded. While not always recommended (George and Sherrington 1984), the solutions containing $HgCl_2$ were more efficient in decontaminating explants. The highest percentage decontamination was obtained by immersion in the ethanol/$HgCl_2$ mixture for 7min (Table 1). All seeds with intact testae were contaminated regardless of how harsh the treatment. Removal of such testae and re-decontaminating the embryos by immersion in the mixture of sterilants for 7min led to 86% decontamination and 45% germination within 30 days. This method was also most effective for the roots, yielding 33% decontaminated and viable explants after 60 days.

While the older explants turned brown on the media, the young leaves and buds remained green. Soaking in citric or ascorbic acid did not remedy this. Leaves placed on media containing 0.3% AC did not turn brown. The leaves, buds and embryos died on 1/2 MS and MS, survived on WPM, but subsequently developed characteristic signs of Ca deficiency. This was rectified by inclusion of 6mM $CaCl_2$ to WPM. New green buds emerged and chlorosis disappeared within 15 days (Figure 1).

Addition of 2,4-D to the medium induced embryos of cv.

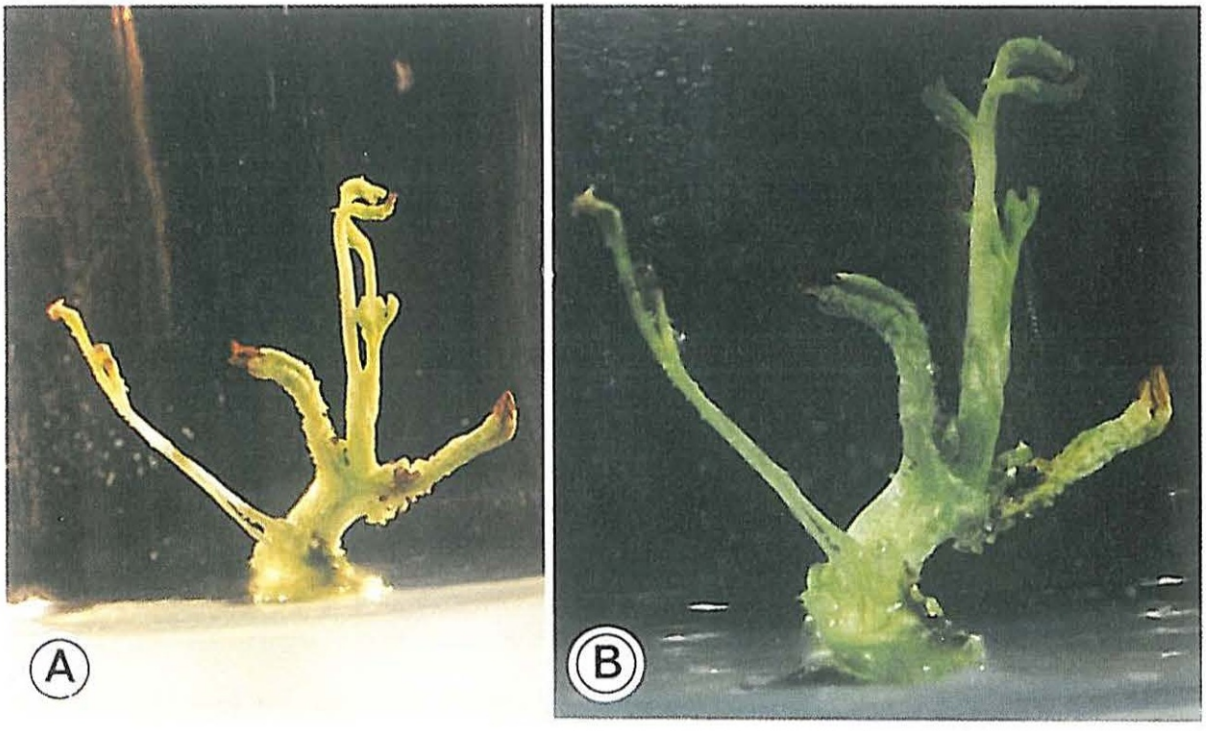

Figure 1: A. Symptoms of Ca deficiency on plantlets cultured on normal WPM (3mM $CaCl_2$) for 60 days. B. New green leaves and axillary buds emerged after 5 days of culture on WPM containing 6mM $CaCl_2$. Ca-deficiency symptoms disappeared from the plantlets after 15 days

'Fengdanbai' to form callus on both ends of the cotyledons (Figure 2A). BA stimulated cotyledon expansion (Figure 2B) while GA_3 promoted embryo elongation on the media containing 1.44 and 2.89µM GA_3 respectively (Figure 2C). Combinations of 2,4-D/BA stimulated cotyledon expansion and callus formation (Figure 2D–G). The four hormone combinations used in an attempt to promote callus growth did not show any major differences in evoking callus formation over a period of 45 days (Figure 3). After this time, however, callus growth significantly improved on a medium containing

Table 1: Decontamination success (%) for leaves (L) and bud (B) explants of *P. suffruticosa* cv. 'Cai Lan', 'Xue Li Zi Yu' and 'Zi Xia Lin'. With 3.5% NaOCl, disinfestation was done at 10°C. The disinfestation mixture contained 20% ethanol and 0.75% $HgCl_2$

		NaOCl (%)			$HgCl_2$ (%)		Mixture	
Time (min)	Explant	0.5	1	3.5	Time (min)	0.1	Time (min)	Sterilant
15	L	4±2	6±3	6±2	3	28±6	5	4±1
	B	–	–	–	–	38±5	–	–
20	L	24±6	12±4	12±3	5	6±2	7	48±7
	B	14±3	–	–	–	–	–	60±8
25	L	6±2	4±2	1±1	7	0	9	0
	B	–	–	–	–	–	–	–

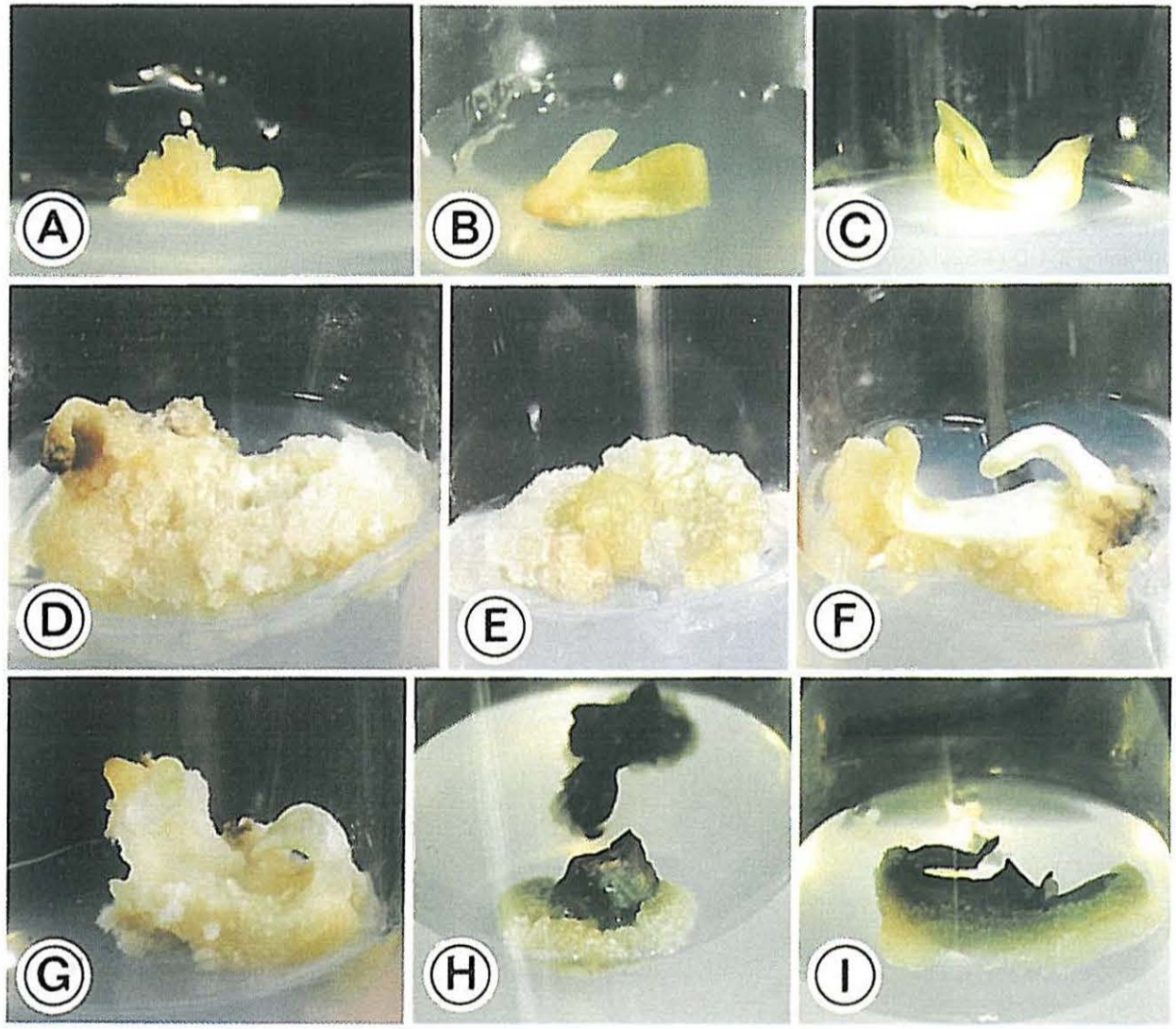

Figure 2: Responses of mature excised embryo axises and cotyledons of *P. suffruticosa* cv. 'Fengdanbai' to phytohormones (A) 4.52µM 2,4-D, (B) 4.44µM BA, and (C) 2.89µM GA_3 introduced to WPM after 35 days of culture. After 60 days of culture, callus proliferated on the media containing 6mM $CaCl_2$ (D) 2.26µM 2,4-D I 2.22µM BA, (E) 4.52µM 2,4-D I 4.44µM BA, (F) 2.26µM 2,4-D I 4.44µM BA, and (G) 4.52µM 2,4-D / 2.22µM BA, respectively. Callus formation from (H) young leaves and (I) petioles of dwarf cultivar 'Cai Lan' on WPM augmented with 4.52µM 2,4-D, 4.44µM BA, and 1.44µM GA_3 for 35 days

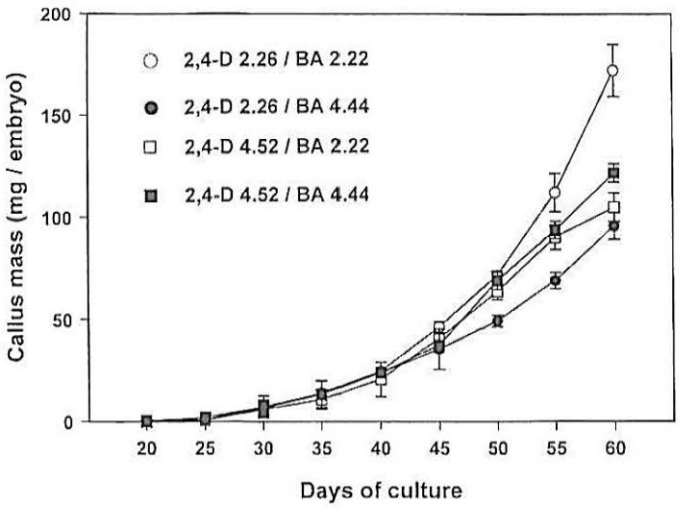

Figure 3: Callus proliferation from the excised mature embryos of *P. suffruticosa* cv. 'Fengdanbai' on WPM containing 6mM $CaCl_2$ supplemented with 2,4-D and BA. Units used were µM. Error bars represent standard error of the mean

2.26µM 2,4-D and 2.22µM BA. The addition of 1.44µM GA_3 to the medium resulted in leaf and petiole pieces expanding and turning green (Figure 2H–I).

Following the original work of Li *et al.* (1984) *in vitro* propagation of tree peonies has been studied frequently. At present, micropropagation does not yet offer a viable commercial option for propagation. Explant contamination and browning are still major problems (Buchheim and Meyer 1992). The present study indicated that by using a mixture of 20% ethanol and 0.075% $HgCl_2$ decontamination can be improved compared to previous results (Gildow and Mitchell 1977, Harris and Mantell 1991, Bouza *et al.* 1994). This study also showed that vigorously growing material could be easily decontaminated and browning contained. The addition of 0.3% AC was effective in preventing tissue and media browning.

In earlier studies (Li *et al.* 1984, Harris and Mantel 1991, Bouza *et al.* 1994, Ikuta *et al.* 1995) MS medium with a high nitrogen content was used. In our studies the dwarf cultivar material died on MS but survived on WPM. The overall growth was improved with the addition of 6mM $CaCl_2$ to this medium. An increased need for higher Ca in the growth media of tree peonies was highlighted earlier. Bouza *et al.* (1984) reported that doubling the Ca concentration resulted in good multiplication rates.

Acknowledgements – Huafang Wang is grateful for a post-doctoral fellowship awarded by NRF, Pretoria.

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Edited by GJ Bredenkamp