Establishment of *in vitro* cultures of tree peonies

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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% HgCl₂ for 7 min. Seeds were best decoted and the embryos then disinfested. The phytohormones 2,4-D, BA and GA₃ 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' 4.52 μM 2,4-D and 4.44 μM BA were required. Addition of 1.44 μM GA₃ 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; GA₃ = gibberelic 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. 'Papaveraceae' (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* (Orlikowska 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 20 min and then decontaminated in groups using three methods: 1) Immersion into 70% (v/v) ethanol for 7–10 sec and then into 0.5, 1.0 and 3.5% NaOCl (active ingredient in commercial bleach) for 15, 20 and 25 min respectively; 2) Immersion into 70% (v/v) ethanol for 7–10 sec and then into 0.1% (v/v) HgCl₂ for 3, 5 and 7 min respectively; 3) Immersion in a mixture of 20% (v/v) ethanol and 0.075% (v/v) HgCl₂ for 5, 7 and 9 min respectively. All explants were subsequently rinsed 3 times (3 min each) in sterile distilled water. Tween 20 (1 drop per 50 ml) was added to each sterilant. In the case of buds, scales were removed whereas after they were treated as the leaves. Roots were heated in water at 42°C for 2 h (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 2 min followed by 30 min in 3.5% (active ingredient) NaOCl or 10 min in 0.1% (v/v) HgCl₂. 2) The seed coat was removed and the embryos were immersed in a mixture of 20% (v/v) ethanol and
0.075% (w/v) HgCl₂ for 7 min; 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 3 mm in length) were excised under aseptic conditions and used; 2.) Leaves and buds were soaked in 0.8 mM ascorbic acid or 0.8 mM citric acid (George and Sherrington 1984) or a combination of these acids (0.4 mM each) for 20 min prior to decontamination. Explants were then inoculated onto a medium containing 0.4 mM 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 (0.8 w/v). On WPM signs of a Ca-deficiency were observed. This was rectified by raising the CaCl₂ level of the medium to 6 mM.

Excised embryos of cv. 'Fengdanbai' were placed on WPM containing 2,4-D (4.52 μM), BA (4.44 μM), or GA₃ (1.44–2.89 μM) 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 6 mM CaCl₂, 500 mg l⁻¹ casein hydrolysate, 1 mM glutamate and combinations of 2,4-D (2.26–4.52 μM) and BA (2.22–4.44 μM). 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 GA₃) were used with the standard WPM. Cultures were maintained at 25±2°C under a 16h/8h light/dark regime. Light (23 μmol m⁻² s⁻¹) was provided by cool white fluorescent tubes supplied by Philips (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 HgCl₂ were more efficient in decontaminating explants. The highest percentage decontamination was obtained by immersion in the ethanol/HgCl₂ mixture for 7 min (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 7 min 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 6 mM CaCl₂ 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 (3 mM CaCl₂) for 60 days. B. New green leaves and axillary buds emerged after 5 days of culture on WPM containing 6 mM CaCl₂. 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 promoted embryo elongation on the media containing 1.44 and 2.89 μM 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 1.44 μM GA$_3$.

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, disinfection was done at 10°C. The disinfection mixture contained 20% ethanol and 0.75% HgCl$_2$.

| Time (min) | NaOCl (%) | HgCl$_2$ (%) | Mixture | Sterilant |
|-----------|-----------|--------------|---------|-----------|
| 15        | L 0.5     | 6±2          | 3       | 28±6      | 4±1       |
| 20        | B 1.0     | 12±3         | 5       | 6±2       | 48±7      |
| 25        | L 3.5     | 6±2          | 7       | 60±8      |           |

Figure 2: Responses of mature excised embryo axises and cotyledons of *P. suffruticosa* cv. 'Fengdanbai' to phytohormones (A) 4.52 μM 2,4-D, (B) 4.44 μM BA, and (C) 2.89 μM GA$_3$ introduced to WPM after 35 days of culture. After 60 days of culture, callus proliferated on the media containing 3 mM CaCl$_2$ (D) 2.26 μM 2,4-D / 2.22 μM BA, (E) 4.52 μM 2,4-D / 4.44 μM BA, (F) 2.26 μM 2,4-D / 4.44 μM BA, and (G) 4.52 μM 2,4-D / 2.22 μM BA, respectively. Callus formation from (H) young leaves and (I) petioles of dwarf cultivar 'Cai Lan' on WPM augmented with 4.52 μM 2,4-D, 4.44 μM BA, and 1.44 μM GA$_3$ for 35 days.

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2.26μM 2,4-D and 2.22μM BA. The addition of 1.44μM GA₃ 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% HgCl₂, 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 Mantell 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 CaCl₂ 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.

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