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Full Length Research Paper

Multiple shoot induction and rooting of *Paeonia lactiflora* 'Da Fu Gui'

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Underground buds of herbaceous peony (*Paeonia lactiflora* Pall.) 'Da Fu Gui' were micropropagated *in vitro*. The basic processes including culture initiation, shoot induction, axillary shoot proliferation and rooting were established. The best initial medium of 'Da Fu Gui' was half-strength MS (Murashige and Skoog) medium (double-strength Ca^{2+}) supplemented with 0.5 mg l^{-1} 6-benzylaminopurine (BA) plus 0.5 mg l^{-1} gibberellic acid (GA_3). The best medium for axillary shoot induction was half-strength MS medium (double-strength Ca^{2+}) supplemented with 1.0 mg l^{-1} BA plus 0.5 mg l^{-1} kinetin (Kin), while 0.5 mg l^{-1} BA + 0.3 mg l^{-1} Kin was best for shoot proliferation. Shoot height at the time of inoculation had a great effect on proliferation and growth of 'Da Fu Gui'. Putrescine (Put) (0.5 to 5.0 mg l^{-1}) prevented rooting of 'Da Fu Gui' but it favored the development of roots. Highest rooting percentage was observed on half-strength MS medium (double-strength Ca^{2+}) supplemented with 1 mg l^{-1} indole-3-butyric acid (IBA).

Key words: Herbaceous peony, underground buds, axillary shoots, micropropagation.

INTRODUCTION

Peonies, of the genus *Paeonia*, are long-lived perennials belonging to Paeoniaceae, having been cultivated in China for more than 3900 years. They can be used as cut, pot or dried flowers or as garden plants. Herbaceous peonies are usually propagated by division of tuberous root clumps containing 3 to 5 dormant vegetative buds (Shannon and Kamp, 1959). However, conventional propagation is too slow to meet the increasing needs of the Chinese and international markets. Tissue culture and rapid propagation of herbaceous peony can raise the propagation rate and shorten the breeding period and this has important significance in the production of popular cultivars or newly selected clones. Many advances have been made in the micropropagation of herbaceous peony. Even so, indirect shoot induction through a callus phase is very difficult and takes a long time while excessive browning is the major problem preventing the formation of shoots from callus (Radtke, 1983). Many studies have focused on indirect shoot induction, but the results were not encouraging. Zhang (2006) induced callus from leaves, stems and petioles of herbaceous peony, but no adventitious shoots were obtained from subcultured callus. According to a recent study, only hypocotyl-derived callus successfully produced adventitious shoots, but at a low regeneration rate (7.95%) (Wang et al., 2010). Direct shoot induction is more prevalent in peony *in vitro* culture with several successful cases reported for both herbaceous peony (Hosoki et al., 1989; Gabryszewska, 1998) and tree peony (Bouza et al., 1994a, b; Kong and Zhang, 1998). Buds, shoot tips, nodal stem, petioles and leaf segments can be used for direct shoot induction; the highest frequency of shoot induction has been found in vegetative axillary buds or

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Abbreviations: MS, Murashige and Skoog**; BA,** 6-benzylaminopurine; **GA_3,** gibberellic acid; **Kin,** kinetin; **IBA,** indole-3-butyric acid; **Put,** putrescine; **2iP,** 6-γ-γ-dimethylaminopurine; **TDZ,** thidiazuron.

shoot tips (Hosoki et al., 1989; Orlikowska et al., 1998; Guo, 2001; Hu et al., 2003; Tian et al., 2010). Many methods were reported to enhance axillary shoot formation, such as the addition of thidiazuron (TDZ) at very low concentrations (0.01 to 0.04 mg l^{-1}) to the medium containing a mixture of 6-benzyladenine (BA) (1, 2 and 4 mg l^{-1}) + 6-γ-γ-dimethylaminopurine (2iP) (1, 2 and 4 mg l^{-1}) + kinetin (Kin) (1, 2 and 4 mg l^{-1}), a longi-tudinal shoot-split method in subculture (Hosoki et al., 1989; Gabryszewska, 1998) and manipulation of the light spectral quality under photoautotrophic conditions (Ding et al., 2010). However, there is still much work to be done, including the application of plant growth regulators (PGRs) at different stages, subculture, rooting and final transplanting, the latter two being large obstacles for the successful micropropagation of herbaceous peony (Guo, 2001; Zhang et al., 2006).

In this experiment, we chose an excellent (highly responsive to manipulation) herbaceous cultivar 'Da Fu Gui', which is a traditional Chinese variety *in vitro* with high ornamental value and vigorous growth *in vitro* (Figure 1A) (Qin, 2004), as the experimental material to induce shoots and to explore the optimum level and combination of PGRs for proliferation and rooting.

MATERIALS AND METHODS

Two-year-old underground buds were collected in spring from the Society of Forestry Experimental Station. The buds were about 3 cm in length and were collected between the closed and sprouting stage.

Surface sterilization

Before inoculating, underground buds were washed in tap water for 30 min. Outer scales were peeled off and denuded buds were soaked in 75% ethanol for 30 s followed by 10 min sterilization with a dilute solution of $HgCl_2$ (0.1% w/v). Buds were rinsed five times (5 min each time) in autoclaved distilled water, then cultured in 100-ml Erlenmeyer flasks containing 30 ml of agar-solidified medium with one bud per flask.

Culture medium

All explants were placed on agar (0.7%) solidified medium. The basal medium for all experiments was half-strength MS medium (1/2 MS; Murashige and Skoog, 1962) with double-strength calcium chloride (Ca^{2+}) supplemented with 30 g L^{-1} sucrose. Double Ca^{2+} was reported to be useful for the growth of both herbaceous peony (Guo, 2001) and tree peony (Bouza et al., 1994a). Different concentrations and combinations of PGRs (all Sigma-Aldrich, St. Louis, USA), including BA, gibberellic acid (GA_3), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), Kin and putrescine (Put) were added to this basal medium.

Culture initiation

The effect of GA_3 (0.5 mg l^{-1}) combined with BA (0.5, 1.0, 1.5 and 2.0 mg l^{-1}) on initiation culture of 'Da Fu Gui' was tested.

Axillary shoot induction

The effect of different treatments on axillary shoot induction was tested. Shoot induction medium was 1/2 MS + 1.0 mg l^{-1} BA, 1/2 MS + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kin, 1/2 MS + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kin + 0.1 mg l^{-1} IBA, 1/2 MS + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kin + 0.1 mg l^{-1} IAA.

Axillary shoot proliferation

There were two experiments. Firstly, the effects of different treatments on shoot proliferation were tested. The treatments were 1/2 MS + 0.5 mg l^{-1} BA + 0.3 mg l^{-1} Kin, 1/2 MS + 0.5 mg l^{-1} BA + 0.3 mg l^{-1} Kin + 500 mg l^{-1} casein hydrolysate (CH), 1/2 MS + 0.5 mg l^{-1} BA + 0.3 mg l^{-1} Kin + 0.2 mg l^{-1} IBA. Then, the effects of initial height of shoots used in inoculation (Figure 1B, C and D) on the proliferation and growth of 'Da Fu Gui' were tested. The shoots were divided into three groups according to height (1, ≤1 cm; 2 = 1 to 2 cm; 3, ≥ 2 cm) before culture. The medium was 1/2 MS supplemented with 0.2 mg l^{-1} BA or PGR-free 1/2 MS medium.

Root induction

In the fifth experiment, the effects of Put (0, 0.5, 1.0, 2.0, 3.0 and 5.0 mg l^{-1}) combined with 1 mg l^{-1} IBA on root induction of 'Da Fu Gui' were tested. At the rooting stage, shoots were placed on different rooting media for 20 days and then transferred to PGR-free 1/2 MS medium containing 0.2% activated charcoal (AC; Sigma). The shoots used for the proliferation and rooting were 2 cm long except shoots for the rooting induction experiment when the effect of Put was 2.0 mg l^{-1}.

Culture conditions

The pH of all media was adjusted to 5.8 prior to autoclaving at 118°C for 18 min. Culture vessels were placed at 25 ± 2°C in a 14-h photoperiod with 50 µmol m^{-2} s^{-1} PPFD using cool white fluorescent tubes. Data were collected after 30 days of culture. The explants were subcultured every 30 days.

Experimental design and statistical analyses

Data was sampled in a completely randomized design. Means were separated by one-way analysis of variance and significant differences were assessed using Duncan's multiple range test at $P = 0.05$ using SPSS software version 13.0. Each experiment contained at least three replications with at least 10 explants per replication. Experiments were conducted on three independent sets of 30 explants per experiment.

RESULTS

The effect of BA on initiation culture

Buds grew rapidly after inoculation. The main shoots and some lateral shoots elongated one week later. Leaves expanded gradually and most explants began to sprout 15 days later. Different concentrations of BA had a significant effect on the percentage shoot initiation, leaf expansion percentage, while the number of lateral shoots formed did not affect plant height (Table 1).

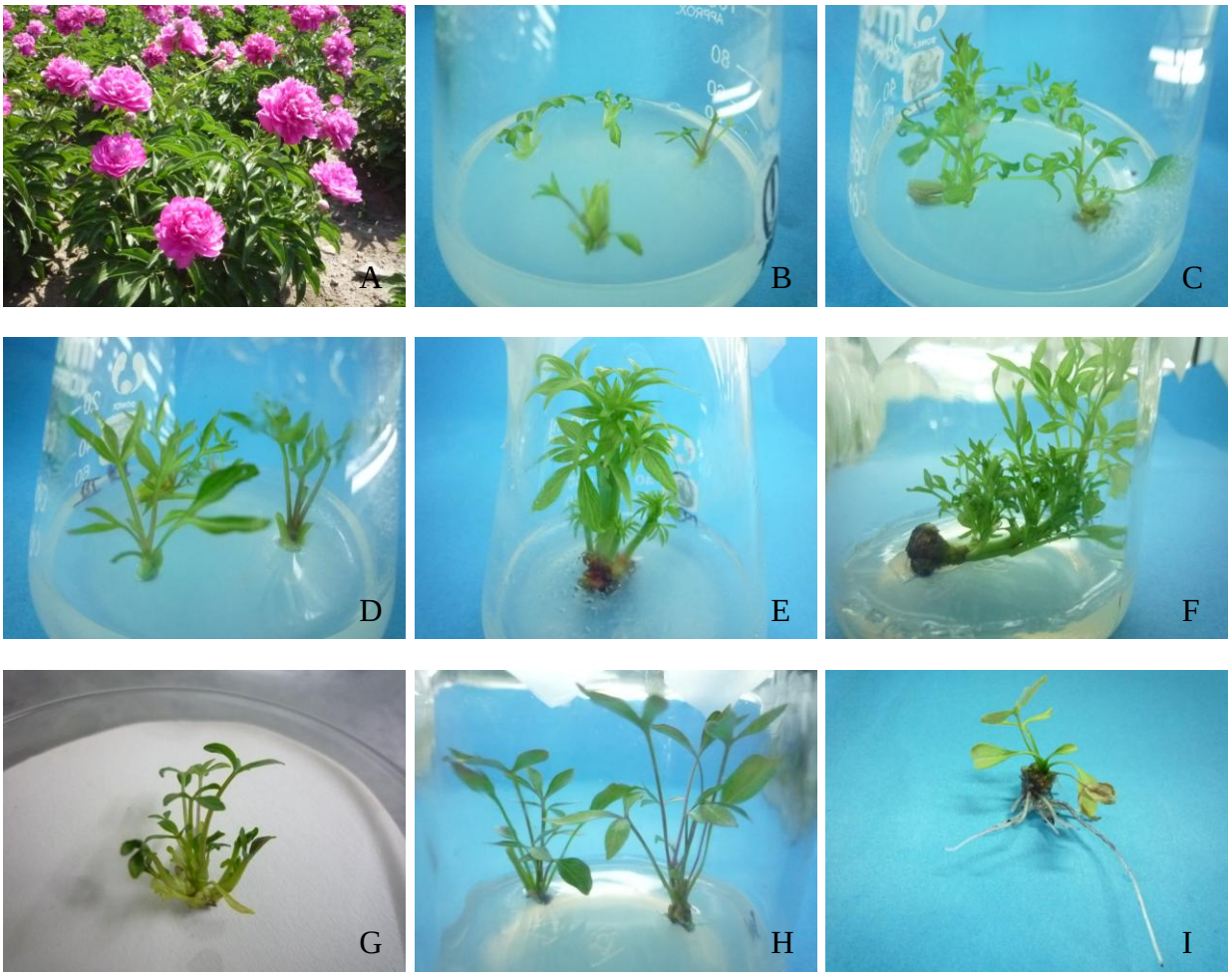

Figure 1. Culture of underground buds of *Paeonia lactiflora* 'Da Fu Gui'. (A) 'Da Fu Gui' flowers; (B) shoots (≤ 1 cm), (C) (1 to 2 cm), (D) (≥ 2 cm) upon inoculation; (E) culture initiation; (F) axillary shoot induction; (G) shoot proliferation; (H) growth of shoots ≥ 2 cm; (I) rooting of plantlets.

The initiation medium with 0.5 to 1.5 mg l^{-1} BA favored the growth of underground buds the most, with a 100% sprouting percentage in all three treatments. The highest leaf expansion percentage (96.97%) and height (3.61 cm) was observed with 1.0 mg l^{-1} BA + 0.5 mg l^{-1} GA_3. The slightly higher number of lateral shoots (2.42) and the best growth of shoots were noted on medium containing the lowest concentration of BA (0.5 mg l^{-1}) (Figure 1E). A higher concentration of BA (2.0 mg l^{-1}) did not further stimulate the growth of shoots, resulting in the lowest sprouting percentage (78.90%), leaf expansion percentage (66.27%) and number of lateral shoots (1.72). In addition, a higher concentration of BA inhibited explant growth, induced hyperhydricity and deformed shoots. The result is consistent with the studies of Wu et al. (2011), who indicated several methods to successfully rejuvenate hyperhydric microshoots, including the addition of 3 g L^{-1} activated charcoal, the removal of ammonium nitrate from the medium, doubling the concentration of Ca^{2+} or eliminating BA from the medium. Thus, medium containing 1.0 mg l^{-1} BA + 0.5 mg l^{-1} GA_3 was the best choice for initial shoot induction of 'Da Fu Gui', considering the higher number (2.28) of lateral shoots.

Axillary shoot induction

Axillary shoots were able to generate after three weeks of culture. Different treatments had a significant effect on axillary shoot induction and necrosis percentage (Table 2). After 20 days of culture, a large number of axillary shoots (about 10) were produced from meristematic regions and from the base of buds (Figure 1F). Medium with 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kin resulted in the highest shoot induction percentage (58.93%). Most axillary shoots (12.29) and the lowest shoot induction percentage (37.30%) were observed with 1.0 mg l^{-1} BA. The addition 0.1 mg l^{-1} IBA or 0.1 mg l^{-1} IAA to medium containing 1.0 mg l^{-1} BA and 0.5 mg l^{-1} Kin decreased the number of axillary shoots. Fewer axillary shoots (7.18) were

Table 1. The effects of various concentrations of BA on initiation culture of 'Da Fu Gui' (n = 10).

Treatment [a] (mg L^{-1})	Germinating rate (%)	Leaf expansion rate (%)	Number of lateral shoots	Height (cm)
0.5 BA	100±0[b]	93.33±11.15[b]	2.42±0.14[b]	3.58±0.12[a]
1.0 BA	100±0[b]	96.97±5.25[b]	2.28±0.10[ab]	3.61±0.20[a]
1.5 BA	100±0[b]	91.07±7.97[b]	2.09±0.50[ab]	3.32±0.24[a]
2.0 BA	78.90±8.11[a]	66.27±8.96[a]	1.72±0.31[a]	3.42±0.12[a]

Different letters within a column indicate significant differences according to Duncan's multiple range test (P = 0.05). [a]Treatment: shoots were inoculated on half-strength MS medium (double-strength Ca^{2+}) supplemented with different PGRs. BA, 6-Benzyladenine; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; Kin, kinetin.

Table 2. The effects of different treatments on axillary shoot induction of 'Da Fu Gui' (n = 10).

Treatment [a] (mg L^{-1})	Induction rate (%)	Death rate (%)	Number of axillary shoots
1.0 BA (control)	37.30±11.25[a]	42.06±8.36[b]	12.29±2.53[c]
1.0 BA + 0.5 Kin	58.93±3.09[b]	22.62±7.43[a]	10.38±0.92[bc]
1.0 BA + 0.5 Kin + 0.1 IBA	40.48±10.91[ab]	45.24±4.12[b]	9.83±1.94[b]
1.0 BA + 0.5 Kin + 0.1 IAA	42.86±14.29[ab]	23.81±8.24[a]	7.18±1.25[a]

Different letters within a column indicate significant differences according to Duncan's multiple range test (P = 0.05). [a]Treatment: underground buds were inoculated on half-strength MS medium (double-strength Ca^{2+}) supplemented with different PGRs. BA, 6-Benzyladenine; GA_3, gibberellic acid.

Table 3. The effects of different treatments on shoot proliferation of 'Da Fu Gui' (n = 10).

Treatment [a] (mg L^{-1})	Multiple
0.5 BA + 0.3 Kin	3.3 ± 0.31[b]
0.5 BA + 0.3 Kin + 500 CH	2.67 ± 0.58[ab]
0.5 BA + 0.3 Kin + 0.2 IBA	2.0 ± 0[a]

Different letters within a column indicate significant differences according to Duncan's multiple range test (P = 0.05). [a]Treatment: shoots were inoculated on half-strength MS medium (double-strength Ca^{2+}) supplemented with different PGRs. BA, 6-Benzyladenine; IBA, indole-3-butyric acid IBA; CH, casein hydrolysate; Kin, kinetin.

observed on medium containing 0.1 mg l^{-1} IAA. In conclusion, medium containing 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kin favored shoot induction the most.

Axillary shoot proliferation

A slightly higher number of multiple shoots (3.3) was obtained on medium with 0.5 mg l^{-1} BA + 0.3 mg l^{-1} Kin (Table 3 and Figure 1G). The fewest multiple shoots were formed on medium with 0.5 mg l^{-1} BA + 0.3 mg l^{-1} Kin + 0.2 mg l^{-1} IBA. The addition of 500 mg l^{-1} CH or 0.2 mg l^{-1} IBA did not favor shoot proliferation. Thus, the best proliferation medium for 'Da Fu Gui' was 0.5 mg l^{-1} BA + 0.3 mg l^{-1} Kin.

Shoot proliferation

Most leaves (7.95), greatest height (2.96 cm) and percentage of sturdy shoots (79.90%) were obtained in shoots taller than 2 cm cultured on PGR-free 1/2 MS medium (Table 4 and Figure 1H). Shoots taller than 2 cm significantly resulted in the best effect followed by shoots 1 to 2 cm in size, then shoots smaller than 1 cm, with or without BA. Shoots taller than 2 cm cultured on 1/2 MS + 0.2 mg l^{-1} BA showed the worst growth parameters. The addition of BA to medium resulted in poor growth of shoots, gradual hyperhydricity and etiolation. Shoots easily became dormant and fragile in summer because of high humidity, so, even a low concentration of BA (0.2 mg l^{-1}) harmed shoot subculture. Thus, during the hot summer months, shoots taller than 2 cm cultured on PGR-free 1/2 MS medium were optimal for subculture.

Rooting

Roots were produced at the base of shoot stems after shoots were transferred to PGR-free 1/2 MS medium containing 0.2% AC for 20 days. The highest rooting percentage (28.15%) was observed on medium with 1.0 mg l^{-1} IBA + 0 mg l^{-1} Put (Table 5 and Figure 1I). Even though the percentage rooting decreased when Put was added, the quality of roots and shoots was good, that is, sturdy and without hyperhydricity. The highest number of roots (4.50), root length (2.07 cm), number of leaves

Table 4. The effect of shoot height on shoot proliferation and growth of 'Da Fu Gui' (n = 10).

Treatment [a]	Multiple shoot	Growth[b] (%)			Height (cm)	Number of leaves
		+	++	+++		
1 (without PGRs)	1.20±0.20[a]	4.50[ab]	34.82[a]	60.68[a]	1.97±0.95[ab]	3.71±1.34[a]
2 (without PGRs)	1.43±0.22[a]	0[a]	36.67[a]	63.34[ab]	2.43±1.08[c]	5.55±2.04[b]
3 (without PGRs)	1.50±0.08[ab]	1.75[a]	18.34[a]	79.90[b]	2.96±1.08[d]	7.95±2.94[c]
1 (0.2 mg L^{-1} BA)	1.24±0.19[a]	19.45[b]	41.40[a]	39.14[a]	1.85±0.87[a]	5.58±1.85[b]
2 (0.2 mg L^{-1} BA)	1.25±0.18[a]	9.70[ab]	33.84[a]	56.46[a]	2.37±1.00[bc]	7.35±2.59[c]
3 (0.2 mg L^{-1} BA)	1.79±0.26[b]	9.51[ab]	38.03[a]	52.46[a]	2.62±1.31[cd]	7.67±3.25[c]

Different letters within a column indicate significant differences according to Duncan's multiple range test (P = 0.05). [a]Treatment: 1: the height of shoots was shorter than 1 cm; 2: the height of shoots was between 1 and 2 cm; 3: shoots were taller than 2 cm. Shoots were inoculated on half-strength MS medium (double-strength Ca^{2+}) supplemented with BA, 6-benzyladenine or without any PGRs. [b] Growth: +++: good growth; ++: average growth; +: poor growth.

Table 5. The effects of various concentrations of putrescine on root inducing of 'Da Fu Gui' (n = 10).

Putrescine (mg L^{-1})	Induction rate (%)	Number of roots	Root length (cm)	Number of leaves	Plant height (cm)
0.0 (control)	28.15±7.18[b]	2.58±0.52[a]	1.88±0.23[b]	4.34±1.14[a]	2.20±0.46[a]
0.5	8.33±8.34[a]	2.00±0.71[a]	0.97±0.68[ab]	4.43±0.15[a]	2.41±0.45[a]
1.0	11.11±4.82[a]	3.00±1.00[a]	1.94±1.23[b]	4.04±0.31[a]	2.65±0.42[a]
2.0	12.12±10.50[a]	4.50±0.50[a]	2.07±0.63[b]	5.05±0.65[a]	2.80±0.35[a]
3.0	6.67±5.77[a]	2.00±1.00[a]	0.51±0.02[a]	3.94±0.52[a]	2.38±0.18[a]
5.0	5.55±4.81[a]	4.00±3.00[a]	1.06±0.34[ab]	4.43±0.30[a]	2.43±0.29[a]

Different letters within a column indicate significant differences according to Duncan's multiple range test (P = 0.05). [a]Treatment: shoots were inoculated on half-strength MS medium (double-strength Ca^{2+}) supplemented with 1.0 mg L^{-1} indole-3-butyric acid (IBA) and different concentrations of putrescine (Put) for 20 days. They were then transferred to PGR-free 1/2 MS medium (double-strength Ca^{2+}) containing 0.2% activated charcoal.

(5.05) and height (2.80 cm) were observed on medium with 1.0 mg l^{-1} IBA + 2.0 mg l^{-1} Put. Higher concentrations of Put (3.0 to 5.0 mg l^{-1}) negatively affected root induction. Put thus does not induce roots but enhances rooting.

DISCUSSION

Considerable research has been conducted on the propagation of peony. Hosoki et al. (1989) used BA (0.5 mg l^{-1}) and GA_3 (1.0 mg l^{-1}) to propagate herbaceous peony cultivars 'Takinoyosooi' and 'Sarah Bernhardt'. Their medium promoted the formation and growth of axillary buds. Yu et al. (2011) indicated the best initial medium for explant establishment of herbaceous peony 'Zhong Sheng Fen' to be half-strength MS medium + (double strength $CaCl_2$) supplemented with 1 mg L^{-1} BA + 0.3 mg L^{-1} GA_3. Wu et al. (2011) found 1 mg L^{-1} BA + 0.5 mg L^{-1} GA_3 + 0.1 mg L^{-1} IAA to most positively influence culture initiation. Gabryszewska (1998) found that a combination of BA (2.0 mg l^{-1}) and TDZ (0.2 mg l^{-1}) did not increase the multiplication rate of herbaceous peony cv. 'Jadwiga', although a combination of various cytokinins (all in mg L^{-1}) {BA (1, 2 and 4), Kin (1, 2 and 4), 2iP (1, 2 and 4) + TDZ (0.01, 0.02 or 0.04)} stimulated axillary bud development. Bouza et al. (1994a) studied the effects of various cytokinins (BA, zeatin, 2iP, iP and KT) and other PGRs on the multiplication of *Paeonia suffruticosa* Andr. cv. 'Mme de Vatry'. Only 1.1 mg L^{-1} BA allowed the development of axillary buds among all the cytokinins tested. Kong and Zhang (1998) found that the addition of a low concentration of auxin (0.1 mg L^{-1} NAA or IAA) to medium containing 1 mg L^{-1} BA increased the number of multiple shoots but stimulated callus formation which was unfavorable for shoot growth in *P. suffruticosa* 'Luo Yang Hong' and 'Yao Huang'. In the present study, half-strength MS medium with 1.0 mg L^{-1} BA + 0.5 mg L^{-1} Kin effectively induced axillary shoots. The addition of 0.1 mg L^{-1} IBA or 0.1 mg L^{-1} IAA to the medium decreased the number of axillary shoots. In axillary shoot proliferation, the addition of CH (>200 mg l^{-1}) or IBA (3.0 mg l^{-1}) did not favor shoot proliferation. The best proliferation medium for 'Da Fu Gui' was 0.5 mg L^{-1} BA + 0.3 mg L^{-1} Kin. The addition of 500 mg L^{-1} CH or 0.2 mg L^{-1} IBA did not favor shoot proliferation. The effect of height of the initial shoot explant on the effectiveness of peony subculture has not yet been reported, although initial

explant size affected the level of callus formation (Wang et al., 2010). Shoots taller than 2 cm resulted in best shoot proliferation in our study.

Polyamines (PAs) play an important role in the development of plants, and are often stress-related (Pang et al., 2007). Although, there are studies that indicate that exogenously-applied PAs are related to rooting *in vitro*, these claims are inconsistent. For example, in walnut, the PAs Put, spermidine and spermine favored rooting and had a synergistic action with IBA; IBA, when applied with spermine, promoted rooting (69%) (Chen, 1994). In contrast, exogenously-applied PAs could not promote rooting of mung bean cuttings, annulling the effect of IBA (Friedman et al., 1982). In this study, the addition of Put to the medium did not favor root induction. PAs are stimulators of roots, callus and shoots in chrysanthemum and tobacco (Teixeira da Silva, 2002).

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