Plant Biotechnology 24, 527–532 (2007)

Tissue Culture Note

Micropropagation and *in vitro* flowering in *Pentanema indicum* Ling

Iyyakkannu Sivanesan[1], Byoung Ryong Jeong[1,2]*

[1] Department of Horticulture, Division of Applied Life Science (BK21), Graduate School, Gyeongsang National University, Jinju 660-701, Korea; [2] Research Institute of Life Science, Gyeongsang National University, Jinju 660-701, Korea

* E-mail: brjeong@gnu.ac.kr Tel: +82-55-751-5489 Fax: +82-55-757-7542

Received January 17, 2007; accepted September 27, 2007 (Edited by S. Ogita)

Abstract In this study we have investigated micropropagation and *in vitro* flowering for a medicinally important plant *Pentanema indicum*. Maximum callus proliferation was obtained on MS medium supplemented with 2.0 mg l^{-1} BAP and 1.0 mg l^{-1} IBA. The best shoot regeneration (19±1.0) was achieved in five weeks when callus was cultured on MS medium amended with 4.0 mg l^{-1} BAP and 1.0 mg l^{-1} IAA. Direct multiple shoot initiation was also obtained from shoot tip and nodal explants in the presence of BAP and IAA. Addition of adenine sulfate (1.0 mg l^{-1}) to the regeneration medium increased the shoot multiplication. Regenerated shoots rooted best on MS medium containing 2.0 mg l^{-1} IBA. Multiple shoots regenerated from callus, shoot tip and nodal explants flowered (90%) *in vitro* on a MS medium fortified with 2.0 mg l^{-1} IBA. Plantlets were successfully acclimatized in a soil condition and the survival rate was 96%.

Key words: Asteraceae, callus culture, direct regeneration, *in vitro* flowering.

Pentanema indicum Ling. belongs to the family Asteraceae (Compositae). It is a female antifertility drug used by tribes in Bihar state of India. The plant is an annual and is distributed throughout India, ascending up to an altitude of 1800 m in Himalayas, Pakistan, Burma, China, Sri Lanka, Thailand and Africa. It is an erect, rigid, leafy herb 1–3 ft in height. The roots are tough, woody and externally pale brown in colour. Stem terete, striate, with many pubescent branches. Leaves are variable in size, sessile, oblong–lanceolate, acute, entire or serrulate, rough or scrabid with short appressed hairs on both sides. Ray florets 12–24, much longer than involcure and disc florets scanty. Many compounds have been isolated from *P. indicum* namely, sesquiterpene–vicolides, monoterpenediol–vicodiol and thymol esters (Saradha Vasanth et al. 1990; Mossa et al. 1997), cis–cis germacranolide (Sawaiker et al. 1998), 4,5,6-trihydroxy-4-7-dimethoxy flavone (Krishnaveni et al. 1997). Vicolide D showed abortifacient and antifertility activities (Alam et al. 1989). The hexane soluble fraction showed antiviral activity against Ranikhet virus (Chowdhury et al. 1990). *In vitro* flowering offers a unique system in the study of molecular basis and hormonal regulation of flowering. There is also a possibility of using *in vitro* flowering in ecological and genetic studies. It is a modern area of research and has great potential in plant breeding programmes. *In vitro* propagation of medicinal plants could help in raising disease free health clones in a large scale for extraction of pure drug. To date only one report available on regeneration for this important medicinal plant (Thulaseedharan and Vaidyanathan 1990) reported callus induction and plant regeneration in *Vicoa indica*. However, regeneration using apical bud and nodal explants derived from mature plants is not available. Therefore, the efficiency of apical bud and nodal explants to regenerate *P. indicum* plants has been explored in the present study. In addition, we have demonstrated a callus mediated plant regeneration system and *in vitro* flowering.

Actively growing shoots were used as the explants source. The shoots were separated from the mother plants, defoliated and cut into 4–6 cm pieces. The explants (leaf and stem) were surface sterilized with 70% (v/v) ethanol for 60 s and 0.1% (w/v) mercuric chloride for 10 min, washed four times with sterile distilled water. Leaf and stem segments 0.5–1.0 cm in length, were prepared and inoculated on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with plant growth regulators. The pH of the medium was adjusted to 5.7 with 0.1 N NaOH or 0.1 N HCl before autoclaving at 1.06 kg cm^{-2} and 121°C for 15 min. All culture media contained 3% (w/v) sucrose and solidified with 0.8% (w/v) agar.

Basal medium supplemented either with BAP or BAP combined with IBA were used for callus induction from leaf and stem explants. The callus induction rate

This article can be found at http://www.jspcmb.jp/

Copyright © 2007 The Japanese Society for Plant Cell and Molecular Biology

(explants with callus formation) was determined after 30 days. After four weeks, the calli proliferated upon the leaf and stem segments. The calli were transferred on MS medium supplemented with different concentrations and combinations of BAP and IAA to induce plant regeneration. Regenerated shoots were dissected out, cut into shoot tips and single nodal segments, and subcultured on MS medium containing different concentration of an auxin and a cytokinin. Multiplication of these shoots was tested in the same media or in media supplemented with adenine sulfate ($1.0\,mg\,l^{-1}$). After four weeks of culture plantlets were transferred to fresh medium containing same composition of ingredients.

Individual shoots, which were 3–4 cm long were excised from the shoot clump and transferred to half strength MS medium containing IAA and IBA (0.5, 10, 1.5 and $2.0\,mg\,l^{-1}$). All cultures were incubated at 25±2°C under cool fluorescent light (3000 Lux; 16-h photoperiod). After 35 days the rooted plantlets were removed from the tubes, washed thoroughly with sterile distilled water to remove traces of agar and planted in small plastic pots filled with mixture of sterile soil, sand, vermiculite (1 : 1 : 1). The potted plants were irrigated with MS basal salts solution (1/4 strength) devoid of sucrose and myo-inositol every four days for three weeks. After three weeks the plants were kept under shade for two weeks and then transferred to glasshouse.

After callus induction, the primary calli were separated from stem explants and they were subcultured on MS medium (3% (w/v) sucrose and $8.25\,g\,l^{-1}$ ammonium nitrate) supplemented with BAP alone or combined with IAA and adenine sulfate. Regenerated shoots of 3–4 cm long were excised and cultured on the MS medium (3% (w/v) sucrose and $8.25\,g\,l^{-1}$ ammonium nitrate) fortified with IAA and IBA. The number of flowering shoots was counted at the end of each subculture period. To study the effects of various carbon source such as glucose, fructose, lactose, maltose, sucrose and nitrogen source viz., ammonium nitrate on *in vitro* flowering. The concentration of each carbon source was 3% (w/v) and ammonium nitrate used was 0, 8.25, 16.5 and $33\,g\,l^{-1}$.

All experiments were repeated thrice. Data were analyzed by analysis of variance (ANOVA) to detect significant differences between means using SAS computer package (SAS Institute Inc., Cary, NC, USA, Release 8.1). Means differing significantly were compared using the Duncan multiple range test at 5% probability level.

Callus was induced from the leaf and stem explants of *P. indicum* on MS medium supplemented with a range of 0.5–$3.0\,mg\,l^{-1}$ BAP either alone or in combination with $1.0\,mg\,l^{-1}$ IBA. The calli were light greenish friable (stem explants) and greenish compact (leaf explants) on medium containing BAP alone, while combination with IBA induced white friable calli in both the explants. The percentage of explants producing calluses as affected by explant type is shown in Table 1. Generally, the highest frequency of callus formation was obtained in stem (92%) and leaf (70%) explants. On increasing the concentration of BAP (1.0–$5.0\,mg\,l^{-1}$), a gradual increase in percentage of culture forming callus was noted (12–41%) in leaf explants. Of the various BAP concentrations, the maximum percentage (63) of light greenish friable callus was obtained from stem explants in medium supplemented with $3.0\,mg\,l^{-1}$ BAP. Higher concentration of BAP inhibited callus induction. The combination of BAP with IBA at the range of 2.0 and $1.0\,mg\,l^{-1}$ respectively induced better proliferation of callus. The primary calli were separated from leaf and stem explants and they were subcultured on MS medium containing either BAP or BAP+IAA. Over a period of 21 days, green compact callus was beginning to turn brown, whereas light greenish friable callus produced reddish or orange pigment. However, the white friable callus multiplication was good and scoring was possible after 60 days of culture. Hence, the white friable callus was used for shoot regeneration and all other callus types were not useful. Calli were small and friable and have always a potential for induction of *de novo* shoot formation. Incorporation of various concentrations of BA in the MS basal medium significantly increased the shoot proliferation rate and number of shoots (data not shown). The shoot primordial started appearing from the white friable callus obtained from the medium containing BAP and IBA on passage to MS medium containing BAP and IAA. The maximum number of shoots (19±1.0) was regenerated from callus cultured on MS medium supplemented with $4.0\,mg\,l^{-1}$ BAP and $1.0\,mg\,l^{-1}$ IAA (Table 2, Figure 1 A and B). Increasing of BA level to $5.0\,mg\,l^{-1}$ reduced the proliferation rate and number of shoots. The number of shoots was reduced to 7.2±1.4 when BAP concentration was decreased to $2.5\,mg\,l^{-1}$. Whereas at low concentrations (1.0 and $2.0\,mg\,l^{-1}$

Table 1. Effect of plant growth regulators on callus induction from leaf and stem explants of *P. indicum*.

Plant growth regulators ($mg\,l^{-1}$)		Callus induction (%) Mean±SD	
BAP	IBA	Stem	Leaf
1.0	—	18.2 ± 1.7^{i}	12.4 ± 0.7^{j}
2.0	—	39.4 ± 1.9^{g}	20.6 ± 1.0^{i}
3.0	—	63.0 ± 1.0^{d}	36.6 ± 1.0^{h}
5.0	—	54.8 ± 1.1^{e}	40.8 ± 1.2^{f}
1.0	1.0	43.1 ± 3.3^{f}	34.0 ± 1.4^{h}
2.0	1.0	92.0 ± 1.0^{a}	64.0 ± 1.0^{d}
2.5	1.0	78.0 ± 1.6^{c}	70.4 ± 1.2^{b}

The experiments were repeated thrice, each experiment consisting of 25 replicates. Results were recorded after 30 days. Variability around the mean was represented as the standard deviation (SD). Mean±SD followed by different letters are not significantly different ($P<0.05$) by Duncan's multiple comparison test.

Copyright © 2007 The Japanese Society for Plant Cell and Molecular Biology

Table 2. Effect of BAP and IAA on shoot regeneration from callus cultures of *P. indicum*.

Plant growth regulators ($mg\,l^{-1}$)		Shoot induction (%) Mean±SD		No of shoots per calli Mean±SD	
BAP	IAA	Leaf callus	Stem callus	Leaf callus	Stem callus
1.0	1.0	—	—	—	—
2.0	1.0	—	—	—	—
2.5	1.0	70.0 ± 1.0^{e}	63.0 ± 2.0^{f}	7.2 ± 1.4^{f}	9.0 ± 1.6^{e}
3.0	1.0	73.0 ± 0.7^{d}	66.4 ± 1.2^{f}	13.9 ± 1.2^{c}	11.4 ± 1.2^{d}
4.0	1.0	89.0 ± 0.2^{a}	77.0 ± 1.0^{c}	19.0 ± 1.0^{a}	14.6 ± 1.4^{c}
5.0	1.0	86.4 ± 1.2^{b}	72.4 ± 1.4^{d}	16.2 ± 1.0^{b}	12.0 ± 1.0^{d}

The experiments were repeated thrice, each experiment consisting of 25 replicates. Results were recorded after 45 days. Variability around the mean was represented as the standard deviation (SD). Mean±SD followed by different letters are not significantly different ($P<0.05$) by Duncan's multiple comparison test.

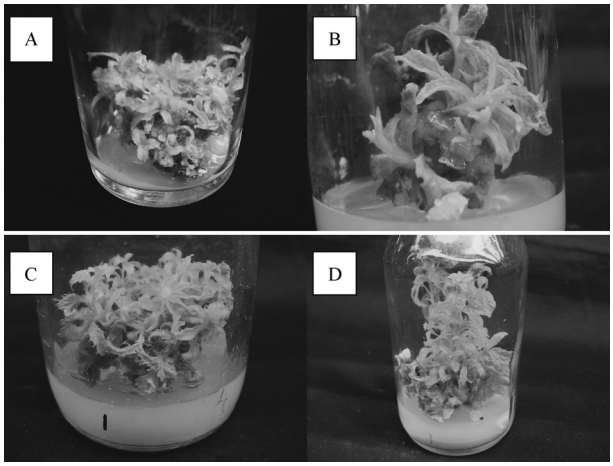

Figure 1. Shoot regeneration from callus, node and shoot tip explants of *P. indicum*. (A) shoot regeneration from stem callus cultures growing on medium supplemented with $4.0\,mg\,l^{-1}$ BAP and $1.0\,mg\,l^{-1}$ IAA. (B) shoot regeneration from leaf callus cultures growing on medium supplemented with $4.0\,mg\,l^{-1}$ BAP and $1.0\,mg\,l^{-1}$ IAA. (C) multiple shoot induction from shoot tip explants cultured on MS medium fortified with $2.0\,mg\,l^{-1}$ BAP, IAA and adenine sulfate each at $1.0\,mg\,l^{-1}$. (D) multiple shoot induction from nodal explants cultured on MS medium fortified with $3.0\,mg\,l^{-1}$ BAP, IAA and adenine sulfate each at $1.0\,mg\,l^{-1}$.

BAP) no shoot formation was observed. Shoot bud regeneration was better in the callus derived from leaf explants as compared to stem explants. The frequency of regenerated shoots varied from 70–89% in leaf and 63–77% in stem derived callus cultures of *P. indicum*. Multiple shoot formation from shoot tip and nodal explants of *P. indicum* was evaluated on MS medium containing different concentrations and combinations of auxin and cytokinin. Among the different explants tested in our study, nodal explants produced greater number of shoots (24). Shoot tip explants produced a maximum of up to 16 shoots at $2.0\,mg\,l^{-1}$ of BAP, IAA and adenine sulfate each at $1.0\,mg\,l^{-1}$ with 100% shoot induction (Figure 1 C). At low concentration of BAP combined with $1.0\,mg\,l^{-1}$ IAA poor shoot induction was observed. Maximum number of shoots were obtained from nodal explants when grown in MS medium supplemented with $3.0\,mg\,l^{-1}$ BAP, IAA and adenine sulfate each at $1.0\,mg\,l^{-1}$ (Figure 1 D). Shoot proliferation and growth decrease were considerably observed with the decrease in BAP level. The addition of $1.0\,mg\,l^{-1}$ adenine sulfate in the media showed increase in shoot proliferation and number of shoots in both nodal and shoot tip explants (Table 3). The number of shoots per explant was higher when subculturing was done on fresh medium at four week intervals on similar medium. The average number of shoot buds per explant increased up to 3–4 fold (based on the explants shoot tip and node respectively) within eight weeks of initial culture which could be maintained for longer periods without any loss in the morphogenetic potential.

Root formation from excised shoots required auxin supplement in the culture medium; roots were visible after 10–14 days, however the results were recorded only after 30 days. Maximum rooting (100%) was obtained on half strength MS medium supplemented with $2.0\,mg\,l^{-1}$ IBA. At $1.0\,mg\,l^{-1}$ IAA induced (86%) rooting. Rooted plantlets were transferred to plastic pots containing sterile soil, sand, and vermiculite (1 : 1 : 1) and kept at 25±2°C and transferred to the glasshouse (after 35 days). The survival rates were as high as 96%.

Flowers initiated in media containing BAP either alone or in combination with IAA. Both BAP and IAA strongly affected the flower bud induction. In the BAP containing medium, there were about 3 buds per culture. There was no bud in the control (MS medium containing 3% (w/v) sucrose and $8.25\,g\,l^{-1}$ ammonium nitrate) cultures. Formation of buds, in general, was greatly enhanced by adenine sulfate. The calli, when subcultured with a few microshoots, continued to produce flowering adventitious microshoots. The production of flowering shoots continued for many subcultures spanning a period of more than two years. Regenerated shoots were excised and cultured on MS medium containing IAA at relatively low concentrations tend to increase the percentage of cultures forming buds. Maximum number of flowers was obtained when the shoots were cultured on MS medium containing $2.0\,mg\,l^{-1}$ IBA (Table 4, Figure 2A–F). Flowering ability increased with the increase in IBA concentration when applied to the plantlets treated

Copyright © 2007 The Japanese Society for Plant Cell and Molecular Biology

Table 3. Effect of plant growth regulators on multiple shoot induction from shoot tip and nodal explants of *P. indicum*.

Plant growth regulators (mg l^{-1})			Shoot induction (%) Mean±SD		No of shoots per node Mean±SD	
BAP	IAA	Adenine sulfate	Node	Shoot tip	Node	Shoot tip
1.0	1.0	—	74.0±3.0^{f}	63.4±1.6^{h}	4.3±1.6^{g}	6.0±0.7^{f}
1.5	1.0	—	80.6±3.7^{e}	68.2±1.4^{g}	4.7±1.2^{g}	8.2±1.2^{e}
2.0	1.0	—	80.0±1.3^{e}	72.0±1.0^{f}	6.0±1.3^{f}	9.6±1.0^{e}
3.0	1.0	—	83.0±1.6^{e}	88.2±1.4^{d}	11.2±2.1^{d}	13.4±1.6^{c}
2.0	1.0	1.0	96.4±1.0^{b}	100^{a}	14.7±3.2^{c}	16.0±0.7^{b}
3.0	1.0	1.0	100^{a}	92.6±2.4^{c}	23.9±2.6^{a}	15.4±1.0^{c}

The experiments were repeated thrice, each experiment consisting of 25 replicates. Results were recorded after 35 days. Variability around the mean was represented as the standard deviation (SD). Mean±SD followed by different letters are not significantly different ($P<0.05$) by Duncan's multiple comparison test.

Table 4. Effect of plant growth regulators on *in vitro* flowering of callus and regenerated shoots of *P. indicum*.

Plant growth regulators (mg l^{-1})				Flower induction (%) Mean±SD		No of flower per shoot Mean±SD	
BAP	IAA	IBA	Adenine sulfate	Callus	RS	Callus	RS
1.0	—	—	—	0	—		—
3.0	—	—	—	13.0±1.0^{i}	—	1.7±0.5^{d}	—
3.0	1.0	—	—	22.8±1.3^{h}	—	2.1±0.7^{d}	—
3.0	1.0	—	1.0	68.0±1.8^{c}	—	3.0±1.0^{c}	—
4.0	1.0	—	1.0	40.0±1.4^{e}	—	4.6±1.4^{b}	—
—	1.0	—	—	—	35.4±1.6^{f}	—	2.8±0.7^{c}
—	2.0	—	—	—	30.0±0.5^{g}	—	3.0±1.0^{c}
—		0.5	—	—	62.6±1.2^{d}	—	2.6±0.7^{cd}
—		1.0	—	—	74.0±1.0^{b}	—	4.0±1.2^{b}
—		2.0	—	—	90.0±1.2^{a}	—	6.0±1.4^{a}
—		5.0	—	—	39.7±1.6^{e}	—	2.3±0.7^{d}

MS medium containing 3% sucrose and half strength ammonium nitrate RS-regenerated shoots (2–3 cm long, devoid of any preformed flower bud). The experiments were repeated thrice, each experiment consisting of 25 replicates. Results were recorded after 6 weeks. Variability around the mean was represented as the standard deviation (SD). Mean±SD followed by different letters are not significantly different ($P<0.05$) by Duncan's multiple comparison test.

previously with BAP+IAA+adenine sulfate. However, high IBA concentrations were ineffective to form reproductive buds. The production of flowers was promoted in approximately the same proportion. Flowering can be induced *in vitro* in excised shoot cultures of *P. indicum* devoid of any preformed bud. The nature of the carbon source (mono or disaccharides) in the medium has a determining influence on the formation of reproductive buds. The carbohydrates differed, in their ability to support the formation of reproductive buds. In general, sucrose was best closely followed by glucose, maltose and fructose were also effective for formation of flowering shoots whereas lactose was totally ineffective. Among the tested ammonium nitrate levels, half strength (8.25 g l^{-1}) induced maximum number of flowering. Increasing the concentration of ammonium nitrate in the medium (1 fold increase) completely inhibited the reproductive bud formation (data not shown).

The appearance of callus varied with both the explants type and the media composition but the response was similar. The callus proliferation obtained with a high concentration of cytokinin and a low concentration of auxin in *P. indicum* was similar to the results obtained by Thulaseedharan and Vaidyanathan (1990). The supply of exogenous growth regulators in the medium was essential for differentiation which otherwise did not occur. Synergistic effect of BAP in combination with IAA on promotion of shoot buds multiplication revealed that in our study two fold enhanced shoot bud proliferation was observed than that in (Thulaseedharan and Vaidyanathan 1990). Proliferation and elongation of adventitious shoots were completed by sub-culturing organogenic calli with shoots in fresh differentiation medium. The determination of organogenesis varied depending on the explants. In this study nodal explants respond well than the shoot tip explants. However, the requirement of plant growth regulator concentration varied among the explants which may be due to the endogenous plant growth regulator concentration. In addition a balance between auxin and cytokinin concentration was also important for shoot bud differentiation. Adenine sulfate which has been found to be effective for bud formation in *P. indicum* also supported the development of floral buds. The highest rate of shoot multiplication was obtained in *Helianthus* (Georgieva et al. 1980) with 0.1 mg l^{-1} BAP, 0.1 mg l^{-1} NAA and 0.1 mg l^{-1} GA3 with the addition of 40 mg l^{-1} adenine sulfate. But

Copyright © 2007 The Japanese Society for Plant Cell and Molecular Biology

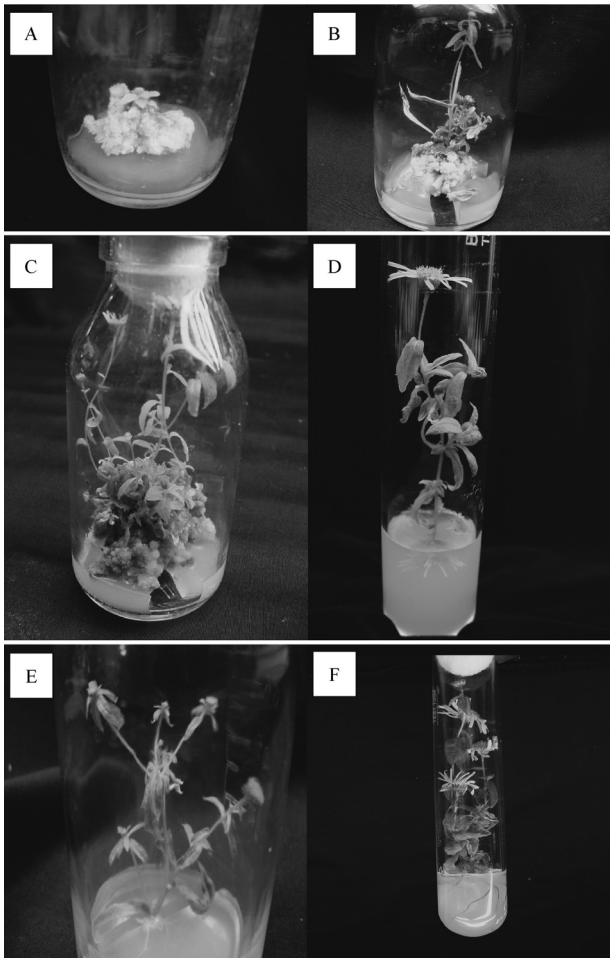

Figure 2. *In vitro* flowering. (A) flower buds initiated from callus cultured on MS medium supplemented with 3.0 mg l^{-1} BAP. (B) flower buds initiated from callus cultured on MS medium containing 3.0 mg l^{-1} BAP and 1.0 mg l^{-1} IAA. (C) flower buds initiated from callus cultured on MS medium supplemented with 3.0 mg l^{-1} BAP, 1.0 mg l^{-1} each IAA and adenine sulfate. (D) flower buds initiated from regenerated shoots cultured on MS medium supplemented with 1.0 mg l^{-1} IAA. (E) flower buds initiated from regenerated shoots cultured on MS medium supplemented with 2.0 mg l^{-1} IBA showing lateral and terminal flower buds. (F) flower buds initiated from regenerated shoots cultured on MS medium containing half strength ammonium nitrate and 2.0 mg l^{-1} IBA.

we report here for the first time that low concentration of adenine sulfate (1.0 mg l^{-1}) was effective for *P. indicum*. Roots were induced (84%) after transferring the shoots to a medium containing low concentration of IBA (0.5 mg l^{-1}) (Thulaseedharan and Vaidyanathan 1990). In contrast, high concentration of IBA induces (100%) rooting. Rooted plantlets were successfully transferred to soil with 96% survival rate. The regenerated plants did not exhibit any detectable variations in morphology or growth characteristics when compared to the respective donor plants. The reaction of flowering depended, to a considerable degree, on the concentration of the applied substances. The plant growth regulator requirement of plants for *in vitro* flowering was variable. In this study, media containing auxins either alone or in combination with BAP induced flowering in callus cultures of *P. indicum*. Similar findings were observed by (Tepfer et al. 1966; Peeters et al. 1994). The essentiality of auxins for flower induction and development has been reported in few plants like *Torenia* (Tanimoto and Harada 1981), *Vigna radiate* (Avendio and Haulea 1990), *Vigna mungo* (Ignacimuthu et al. 1997), *Pisum sativum* (Franklin et al. 2000), and *Perilla frutescens* (Zang 2007). In the present investigation IBA induce maximum number of reproductive buds (Figure 2E and F). Flowering ability increased with the increase in IBA concentration applied to the regenerated shoots which were separated from BAP+IAA+adenine sulfate grown media. A beneficial effect of purines and pyrimidines on flower induction has been reported by Chailakhyan et al. (1961) in the case of *Perilla nankinensis* cultured *in vitro*. Our observation coincides with the above report in that the suitable level of adenine and BAP is beneficial for flower formation. It was found that *in vitro* flowering was also influenced by the levels and ratios of the two major components, carbohydrates and minerals. The presence of a carbohydrate source is a ubiquitous requirement for reliable induction and development of flowers *in vitro* (Scorza 1982). Flowering of *P. indicum* is dependent on the presence of a carbohydrate source. In the present investigation sucrose was found to be the best for flower bud induction. High frequency of flowering was observed when the medium was supplemented with 3% (w/v) sucrose; this result coincides with earlier reports in *Lycopersicon esculantum* (Rastogi and Sawhney 1987), in potato (Al-Wareh et al. 1989), in *V. mung*o (Ignacimuthu et al. 1997), in *P. sativum* (Frankiln et al. 2000), and in *Gentiana trifolia* (Zang and Leung 2002). According to the floral nutrient diversion hypothesis, C/N ratios increase in buds during flower induction (Sachs 1977). In the present investigation the MS medium supplemented with 3% (w/v) sucrose and plant growth regulators produced few flowering shoots. This result shows that the C/N ratio is important for flowering. Many studies have reported the effect of nitrogen level on *in vitro* flowering (Tanimoto and Harada 1981; Narasimhulu and Reddy 1984; Dickens and Van-Staden 1988; Duan and Yazawa 1994; Kostenyuk et al. 1999; Dielen et al. 2001).

In this study, number of flower and percentage of flower bud induction were consistently increased in shoots culturing onto MS medium containing half strength ammonium nitrate. A similar finding was observed by Ignacimuthu et al. (1997) in *V. mungo*, Franklin et al. (2000) in *P. sativum* and Zang (2007) in *P. frutescens*. High concentrations of nitrogen in MS media inhibited flowering and promoted vegetative growth, which competed more efficiently for carbohydrates from the medium (Dielen et al. 2001). In this study,

Copyright © 2007 The Japanese Society for Plant Cell and Molecular Biology

high concentration of ammonium nitrate was not suitable for shoot bud development also, strongly inhibiting flowering. These results suggest that ammonium nitrate concentration is one of the important factors regulating *in vitro* flowering in *P. indicum*. The malformation and poor flower quality occasionally observed in the flowers produced *in vitro* may have been atleast partially due to competition and/or nutritional deficiencies. The flowering plantlets described in the present study may have a practical value in wide hybridization and *in vitro* fertilization studies, especially in studying the mechanism of fertilization of *P. indicum* under experimental condition because *in vitro* flowering of regenerated plantlets can be induced at any time of the year. We have reported here very simple and efficient protocol for plant regeneration and *in vitro* flowering as compared to the methods described for other members of Asteraceae.

Acknowledgements

Iyyakkannu Sivanesan was supported by a scholarship from the BK21 program, Ministry of Education and Human Resources Development, Korea.

Reference

Alam MM, Susan T, Joy S, Bhima Rao R (1989) Antifertility and abortifacient potentiality of vicolide D. *Indian Drugs Pharm Industries* 26: 337–340

Al-wareh H, Trolinder NL, Goodin JR (1989) *In vitro* flowering of potato. *Hort Sci* 24: 827–829

Avenido RA, Haulea DM (1990) *In vitro* organogenesis and flowering in mungbean (*Vigna radiate* L.). *Phil J Crop Sci* 15: 169–173

Chailakhyan MKH, Butenko RB, Lyubarskaya II (1961) Effect of derivatives of nuclei acid metabolism on the growth and flowering of *Perilla nankinensis*. *Fiziol Rast* 8: 101–113

Chowdhury BL, Hussaini FA, Shoeb A (1990) Antiviral constituents of *Vicoa indica*. *Int J Crude Drug Research* 28: 121–124

Dickens CWS, Van-Staden J (1988) *In vitro* flowering of *Kalancheo blossfeldiana* Poellniz. 1. Role of culture conditions and nutrients. *J Exp Bot* 39: 461–471

Dielen V, Lecouvet V, Dupont S, Kinet JM (2001) *In vitro* control of floral transition in tomato (*Lycopersicon esculentum* Mill.) the model for autonomously flowering plants using the late flowering *uniflora* mutant. *J Exp Bot* 52: 715–723

Duan JZ, Yazawa S (1994) *In vitro* floral development in X Doriella Tiny (*Doritis pulcherima*×*Kingella philippinensis*). *Scientia Horticulturae* 59: 253–264

Franklin G, Pius PK, Ignacimuthu S (2000) Factors affecting *in vitro* flowering and fruiting of green pea (*Pisum sativum* L.). *Euphytica* 115: 65–73

Georgieva-Todorova J, Bohorova N, Atanassov A (1980) *Proceedings of the 9th International sunflower conference*, Torremolinos, Spain, 122–128

Ignacimuthu S, Franklin G, Melchias G (1997) Multiple shoot formation and *in vitro* fruiting of *Vigna mungo* L. Hepper. *Curr Sci* 73: 733–735

Kostenyuk I, Oh B J, So I S (1999) Induction of early flowering in *Cymbidium niveo-marginatum* Mak. *Plant Cell Rep* 19: 1–5

Krishnaveni M, Suja V, Vasanth S, Shymala devi CS (1997) Anti-inflammatory and analgesic actions of 4,5,6-trihydroxy-3-7-dimethoxy flavone from *Vicoa indica* DC. *Indian J Pharmacology* 29: 178–181

Mossa JS, EI-Feraly FS, Muhammed I, Zew K, Mbwambo ZH, Pezzuto JM, Fong HHS (1997) Sesquiterpene lactones and thymol esters from *Vicoa pentanema*. *J Natural Product* 60: 550–555

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473–497

Narasimhulu SB, Reddy GM (1984) *In vitro* flowering and pod formation from cotyledons of groundnut (Arachis hypogeaea L.). *Theor Appl Genet* 69: 87–91

Peeters AJM, Proveniers M, Hoek AV, Schreuder M, Gerards W, Barendse GWM, Wullems GJ, Van-Hoek A (1994) Isolation and characterization of mRNA accumulated during *in vitro* flower bud formation. *Planta* 195: 271–281

Rastogi R, Sawhney VK (1987) The role of plant growth regulators, sucrose and pH in the development of floral buds of tomato (*Lycopersicon esculentum* Mill.) cultured *in vitro*. *J Plant Physiol* 128: 285–295

Sachs RM (1977) Nutrient diversion: An hypothesis to explain the chemical control of flowering. *Hort Sci* 12: 220–222

Saradha Vasanth, Kundu AB, Purushothaman KK, Patra A, Vasantha Pattabhi, Connolly JD (1990) Isolation and characterization of vicodiol a new monoterpenediol from *Vicoa indica* DC. *J Natural Product* 53: 354–358

Sawaiker DD, Rojatkar SR, Nagasampagi BA, Puranik VG (1998) A germacranolide from *Vicoa indica*. *Phytochemistry* 48: 515–518

Scorza R (1982) *In vitro* flowering. *Hort Rev* 4: 106–127

Tanimoto S, Harada H (1981) Effects of IAA, zeatin, ammonium nitrate and sucrose on the initiation and development of floral buds in *Torenia* stem segments cultured *in vitro*. *Plant Cell Physiol* 22: 1553–1560

Tepfer SS, Karpoef AJ, Greyson RI (1966) Efects of growth substances on excised floral buds of *Aquilegia.* *Amer J Bot* 53: 148–157

Thulaseedharan A, Vaidyanathan CS (1990) Induction of callus and plant regeneration in *Vicoa indica*. *Plant Cell Tiss Org Cult* 23: 45–48

Zhang T (2007) *In vitro* flowering of *Perilla frutescen*s. *In vitro Cell Dev Biol Plant* 43: 91–94

Zhang Z, Leung DWM (2002) Factors influencing the growth of micropropagated shoots and *in vitro* flowering of gentian. *Plant Growth Regul* 36: 245–251

Copyright © 2007 The Japanese Society for Plant Cell and Molecular Biology