Indian Journal of Agricultural Sciences **83** (9): 912–7, September 2013/Article

Efficient plant regeneration and *in vitro* rhizome induction of manyflower betony (*Stachys floridana*)

JUAN LI[1], GUOYU ZHANG[2], ZHIHUI CHENG[3] and WEIFU LIN[4]

College of Horticulture, Northwest Sci-Tech University of Agriculture and Forestry, Yangling, Shanxi 712 100, China

Received: 15 May 2012; Revised accepted: 7 January 2013

ABSTRACT

Manyflower betony (*Stachys floridana* Schuttl ex Benth) is a specific vegetable variety in China grown for rhizomes. An efficient protocol was developed for plantlet regeneration and *in vitro* rhizome induction to facilitate germplasm conservation, rapid propagation as well as breeding and production of this species. With this regeneration system, 79.8% of explants produced an average of 5.4 elongated shoots per explant, and 100% of shoots produced roots within 15 days. We also reported an efficient method for rapid *in vitro* rhizome induction from regeneration plantlets. This simple and efficient plant regeneration and *in vitro* microrhizome induction system could be useful for propagation of this vegetable and a promising approach to produce metabolites of interest, such as stachyose.

Key words: *In vitro* rhizome induction, Leaf explants, Manyflower betony, Micropropagation

Manyflower betony (*Stachys floridana* Schuttl.ex Benth) is an annual herb vegetable species, indigenous to China and has now been naturalized in other parts of the world such as Japan, Europe and America (Zhong *et al.* 2006a). The root stems of manyflower betony can be eaten and usually is enjoyed as an appetizer in the meal due to its nutritious value and good flavour. Currently, it is one of the special food for Chinese astronaut (Zhong *et al.* 2006b). Manyflower betony contained abundant stachyose, vitamin C and amino acid (Zhong *et al.* 2006a). Stachyose has the function to soften the blood vessel, improve the blood cycling, resist the diseases from intestines, inhibit the development of hypertension and prevent the dementia of old people (Hayashi *et al.* 1994, Huang *et al.* 2006, Zhao and Yang 2007). Nowadays, stachyose in the market is mainly coming from corns or soybeans (Nakakuki 2002). However, these products are not pure enough (Conkerton *et al.* 2006). Therefore, the stachyose coming from the rhizome of manyflower betony has remarkable advantages owing to its purity (Zhong *et al.* 2006a).

Manyflower betony is propagated traditionally by rhizome sprouting. However, the seed rhizomes always have delayed germination and have a very low germination rate, which makes its germplasm preservation and propagation difficult (Zhang *et al.* 2003). In addition, the seed rhizomes are difficult to store, they often die after desiccation. To overcome these problems, investigating an efficient micropropagation method for manyflower betony has become necessary. *In vitro* culture methods for adventitious bud production have been developed for several *Stachys* species (Li *et al.* 2002, Legkobit and Khadeeva 2004).

In this study, culture parameters including the types and concentration of plant growth regulator, sucrose concentration, incubation temperature and photoperiod were evaluated. An efficient protocol for shoot regeneration from leaf explants, and *in vitro* rhizome induction from regenerated plantlets were developed. Micropropagation can enhance propagating capacity and supply many seedlings for large-scale planting. Furthermore, *in vitro* microrhizome induction act as bioreactors, a promising approach to produce metabolites of interest, such as stachyose.

MATERIALS AND METHODS

Plants of manyflower betony (*Stachys floridana* var. *erxiyicu*) grew in a walk-in plant growth chamber (model

[1] Assistant Professor (e mail: njtrs2003@163.com), Rubber Research Institute, Chinese Academy of Tropical Agricultural Sciences, Danzhou, Hainan 571737, China; [2] Associate Professor (e mail: zhgy95@163.com), Beijing Vegetable Research Center, Beijing Academy of Agriculture and Forestry Sciences,Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (North China), Ministry of Agriculture, Key Laboratory of Urban Agriculture (North), Ministry of Agriculture, Beijing 100097, China; [3] Professor (e mail: chengzh@nwsuaf.edu.cn), College of Horticulture, Northwest Sci-Tech University of Agriculture and Forestry, Yangling, Shanxi 712100, China; [4] Professor (e mail: rubberl@163.com), Rubber Research Institute, Chinese Academy of Tropical Agricultural Sciences, Danzhou, Hainan 571737, China

PGV36; Conviron Systems of America, Pembina, ND) at College of Horticulture, Northwest Sci-Tech University of Agriculture and Forestry were used in this study. Fully-expanded young leaves of the 15- to 20-cm tall plants of the manyflower betony were used as explants.

Harvested leaves were excised into 1 cm × 2 cm and surface disinfected in 70% (v/v) ethanol for 30 s, then in sodium hypochlorite (0.5%, w/v) and a few drops of Tween 20 for 7 min, followed by four rinses in sterile, deionized water and cut into 0.3 to 0.5 cm^2 explants, then were placed horizontally in Erlenmeyer flasks containing 30 mL of Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 30 g/L sucrose, 1.0, 2.0, 3.0, 4.0, or 5.0 mg/L Benzylaminopurine (BA) in combination with 0.1, 0.2, 0.5, or 0.8 mg/L 1-naphthaleneacetic acid (NAA), or 0.01, 0.1, or 0.5 mg/L thidiazuron (TDZ) in combination with 0.5 mg/L NAA, and solidified with 7 g/L agar (Eiken, Tokyo, Japan). The pH of the medium was adjusted to 5.8 then autoclaved at 121 °C for 20 min. The experiment was replicated four times with 20 explants per treatment, per replicate. The cultures were incubated at 25 °C under a 16-hr photoperiod provided by cool-white fluorescent lamps (60 μmol/m^2/s) and transferred to fresh media every 14 d. After 30 d, the percentage of bud regeneration, the number of buds per explant and the length of buds were recorded.

After 30 d of induction culture, the leaf explants with buds were transferred onto the shoot elongation medium consisting of MS medium supplemented with 30 g/L sucrose, 2.0 mg/L BA, 0.5 mg/L NAA, and 0, 0.5, 1.0, or 2.0 mg/L gibberellic acid (GA_3). After 20 d, the length of normal elongated shoots per explant was measured.

Elongated shoots, about 2.0 to 3.5 cm in length from the leaf explants, with at least two expanded leaves were excised and rooted on root-induction medium, which consisting of MS medium supplemented with 30 g/L sucrose, 0, 0.1, 0.5, 1.0, 1.5 mg/L NAA, or 0.1, 0.5, 1.0, 1.5 mg/L indole-3-acetic acid (IAA), respectively. Number of roots and root length were recorded after 20 d.

After 10 d on rooting medium, rooted plantlets were transplanted into plastic pots containing moist, autoclaved substrate composed with perlite and vermiculite (1/1, v/v). The pots were covered with plastic film to provide a high relative humidity. Potted plants were placed in growth chambers under a 16-hr photoperiod (65.5 μmol/m^2/s) and 25 °C. Plantlets were watered weekly, and gradually acclimatized (over a period of 2 weeks) to room temperature and humidity conditions by progressively opening the plastic film until plants were ready for transfer to the greenhouse. In the greenhouse, the plantlets were repotted in big pots and watered as needed to avoid water stresses. In mid-April plantlets were transplanted to field conditions.

The plantlets initiated from the leaf explants with 5 to 6 nodes were further used for rhizome induction experiments. Shoot segments (1–1.5 cm) containing one node and two leaves were excised from the aseptically plantlets and were vertically inserted into rhizome induction medium with the upper part upward. Four kinds of rhizome induction media were used, as follows: 1) MS medium supplemented with 10% sucrose; 2) MS medium supplemented with 1.0, 3.0, 5.0, or 10.0 mg/L BA and 10% sucrose; 3) MS medium supplemented with 5.0 mg/L BA and 500 mg/L (2-chloroethyl) trimethylammonim chloride (CCC) and 10% sucrose; 4) MS medium supplemented with 5.0 mg/L BA and 500 mg/L CCC and 3% sucrose. Temperature (10, 15, 20, 25 °C) and photoperiod (0-hr, 8-hr and 14-hr photoperiod) treatment on rhizome induction were also investigated here. After 42 d, the number of rhizomes per plantlet and length and fresh weight of rhizome were investigated.

Analysis of variance (ANOVA) was used to test the statistical significance, and the significance of differences among means was carried out using Duncan's (1955) multiple-range test at the 5% level.

RESULTS AND DISCUSSION

Buds regeneration from leaf explants

Callus began to form on the wounded margins of leaf explants at 10 d after the explants were placed on MS medium supplemented with BA and NAA, or TDZ and NAA, with single shoot bud or bud clusters subsequently formation after 30 d (Fig 1 A). Percentage of bud regeneration, the mean number of buds per explant and the length of buds varied from 0% to 79.8%, 0 to 5.4 and 0 cm to 0.72 cm, respectively, with different medium. The highest percentage of bud regeneration (79.8 ± 4.25%), the highest mean number of buds per explant (5.4 ± 0.35) and the highest length of buds (0.72 ± 0.03 cm) occurred on MS medium supplemented with 2.0 mg/L BA plus 0.5 mg/L NAA (Table 1).

The effect of BA on the buds regeneration depended strongly on the concentration of NAA. At lower BA concentration (≤ 2 mg/L), the percentage of bud regeneration, the number of buds per explant increased significantly with the increase of NAA concentration except for treatment with NAA 0.8 mg/L. Higher BA concentration (≤ 3 mg/L) show decreased ability in shoot induction. Medium with TDZ also generated relatively high number (3.5 ± 0.10) of buds, but TDZ inhibited bud elongation and decreased the average percentage of bud regeneration (Table 1).

Shoots elongation

Bud clusters were transferred into different shoot elongation medium with varied GA_3 concentration. GA_3 has significant effects on the length of shoots (Table 2). Adventitious buds grew quickly, and the shoots were normal and strong on MS medium supplemented with 2.0 mg/L BA, 0.5 mg/L NAA, and 0.5 mg/L GA_3 (Fig 1 B).

Table 1 Effect of plant growth regulators on adventitious buds regeneration from leaf explants

Plant growth regulators(mg/L)			Bud regeneration (%)	Mean number explant buds/ explant	Length of buds (cm)
BA	TDZ	NAA			
1.0		0.1	0 l	0 l	0 k
1.0		0.2	10.8 ± 0.33 k	2.6 ± 0.09 f	0.42 ± 0.02 f
1.0		0.5	26.2 ± 0.59 hi	3.4 ± 0.18 de	0.38 ± 0.01 g
1.0		0.8	23.5 ± 0.45 i	4.0 ± 0.06 c	0.41 ± 0.01 f
2.0		0.1	41.6 ± 1.45 e	2.7 ± 0.07 f	0.50 ± 0.02 cd
2.0		0.2	50.4 ± 2.15 d	3.3 ± 0.15 e	0.52 ± 0.03 c
2.0		0.5	79.8 ± 4.25 a	5.4 ± 0.35 a	0.72 ± 0.03 a
2.0		0.8	71.0 ± 3.48 b	4.6 ± 0.13 b	0.65 ± 0.01 b
3.0		0.1	60.8 ± 2.74 c	4.1 ± 0.08 c	0.46 ± 0.02 e
3.0		0.2	52.6 ± 2.49 d	2.0 ± 0.08 g	0.32 ± 0.02 h
3.0		0.5	40.2 ± 2.51 ef	0.9 ± 0.05 i	0.43 ± 0.01 f
3.0		0.8	37.6 ± 1.68 f	1.0 ± 0.05 i	0.48 ± 0.02 de
5.0		0.1	40.2 ± 1.69 ef	0.3 ± 0.02 k	0.50 ± 0.02 cd
5.0		0.2	28.2 ± 0.88 gh	0.6 ± 0.04 j	0.31 ± 0.02 h
5.0		0.5	20.4 ± 0.91 j	0.4 ± 0.03 k	0.36 ± 0.01 g
5.0		0.8	19.35 ± 0.90 j	0.7 ± 0.04 j	0.37 ± 0.03 g
	0.01	0.5	30.2 ± 0.93 g	1.3 ± 0.05 h	0.32 ± 0.01 h
	0.1	0.5	25.4 ± 0.73 hi	3.3 ± 0.19 e	0.27 ± 0.01 i
	0.5	0.5	10.2 ± 0.40 k	3.5 ± 0.10 d	0.22 ± 0.02 j

Values represent means ± standard deviation. Means within a column followed by the same letter are not significantly different by Duncan's multiple range test (DMRT) at 0.05 probability level.

Values represent means ± standard deviation. Means within a column followed by the same letter are not significantly different by Duncan's multiple range test (DMRT) at 0.05 probability level.

Rooting and transplanting

Regenerated shoots (2.0 to 3.5 cm in height) were excised from bud clusters for rooting. Healthy and strong roots were observed after 5 d on the medium supplemented with 0.5 mg/ L NAA (Fig 1 C and D). In medium supplemented with IAA, rooting initiated at day 7. In medium without auxin, rooting initiated at day 9 and the roots were relatively slender and fragile. Auxin NAA is better than IAA in root induction. It should be noted that auxin decreased the root length, treatment

Table 2 Effect of plant growth regulators on shoot elongation

Plant growth regulators (mg/L)			Shoot regeneration (%)	Mean length of shoot (cm)
BA	NAA	GA3		
2.0	0.5	0	100	0.72 ± 0.05 d
2.0	0.5	0.5	100	3.31 ± 0.31 a
2.0	0.5	1.0	100	2.75 ± 0.16 b
2.0	0.5	2.0	100	2.41 ± 0.23 c

Table 3 Effect of auxin type and concentration on root formation.

Auxin (mg/L)		Number of roots/ shoots	Length of root	Root formation (%)
NAA	IAA			
0	0	6.3 ± 0.4 e	1.12 ± 0.09 a	100
0.1		9.6 ± 1.0 d	0.87 ± 0.16 b	100
0.5		13.0 ± 1.0 a	0.78 ± 0.08 b	100
1.0		11.6 ± 0.9 bc	0.49 ± 0.04 cd	100
1.5		12.4 ± 0.7 ab	0.37 ± 0.06 d	100
	0.1	7.3 ± 0.5 e	0.92 ± 0.21 b	100
	0.5	10.0 ± 1.2 d	0.79 ± 0.08 b	100
	1.0	10.6 ± 0.6 cd	0.56 ± 0.08 c	100
	1.5	11.3 ± 0.8 bc	0.42 ± 0.08 cd	100

Values represent means ± standard deviation. Means within a column followed by the same letter are not significantly different by Duncan's multiple range test (DMRT) at 0.05 probability level.

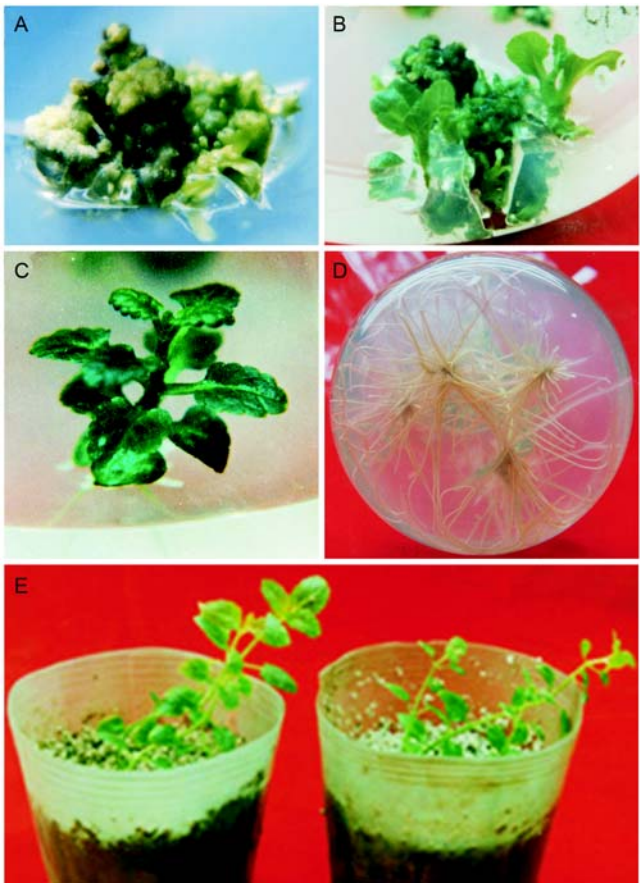

Fig 1 Plants regeneration from leaf explants of manyflower betony. A, Callus induction and multiple adventitious buds emergence from cultured leaf explants; B, Buds developed into shoots on elongation medium; C, A regenerated plantlet; D, Development of roots on root induction medium; E, Acclimatized plants were transplanted into soil.

without auxin gave the highest values of average root length with 1.12 cm (Table 3).

Three hundred and eighty-six rooted plantlets were transplanted in pots, and 92.5% of plants survived in greenhouse conditions after 42 d of acclimatisation tage (Fig 1 E). There was no detectable variation among the acclimatized plants with respect to morphological and growth characteristics.

Effect of sucrose, BA and CCC on rhizome induction

The regenerated plantlets were used for further *in vitro* rhizome induction and the results show that sucrose play a crucial role in rhizome formation (Table 4). The rhizome was barely produced with lower sucrose concentration (= 5%). BA can also facilitate *in vitro* rhizome formation, and 5.0 mg/L BA was suitable (Table 4). The average rhizome number and the fresh weight of rhizome per plantlet increased slightly with the concentration of BA increased, whereas the average rhizome length and the fresh weight of individual rhizome decreased. With the concentration of BA increasing from 1.0 mg/L to 10.0 mg/L, the average rhizome number and the fresh weight of rhizome per plantlet increased by 39.1% and 21.5%, whereas the rhizome length and the fresh weight decreased by 19.8% and 15.4%, respectively. The fresh weight of rhizome per plantlet increased with the concentration of BA enhancement mainly due to the increase of the rhizome number. The medium supplemented with 500 mg/L CCC could enhance the rhizome length and the fresh weight. Taken together, the MS medium supplemented with 10% sucrose, 5.0 mg/L BA and 500 mg/L CCC was optimal for rhizome formation.

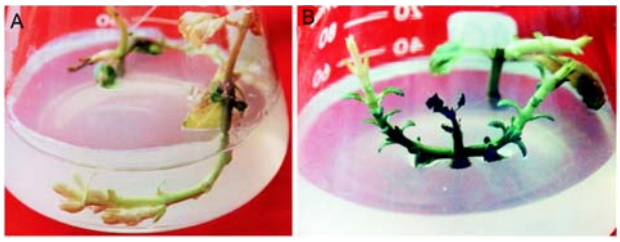

Fig 2 *In vitro* rhizome induction of manyflower betony. A, Rhizome formation under complete darkness. B, Axillary buds developed into lateral branches instead of rhizome formation under illumination condition (8 hr/d or 14 hr/d).

Effect of temperature and photoperiod on rhizome induction

Complete darkness was essential for rhizome formation. The axillary buds from explants grew bend downwards into medium to form rhizomes under complete darkness condition (Fig 2A). However, they grew upwards and gradually develop into lateral branches under the illumination condition, regardless the short (8 hr/d) or the long photoperiods (14 hr/d) (Fig 2B).

Table 4 Effect of sucrose, plant growth regulators and temperature on *in vitro* rhizome induction.

Treatment	Number of the induced rhizome per plantlet	Length of rhizome (cm)	Fresh weight of individual rhizome (g)	Fresh weight of rhizome per plantlet (g)
Sucrose (%)				
2	0.00 c	0.00 c	0.000 c	0.000 c
3	0.00 c	0.00 c	0.000 c	0.000 c
5	0.00 c	0.00 c	0.000 c	0.000 c
8	1.00 ± 0.11 b	2.91 ± 0.12 b	0.273 ± 0.015 b	0.271 ± 0.022 b
10	1.21 ± 0.13 a	3.16 ± 0.13 a	0.294 ± 0.009 a	0.356 ± 0.017 a
BA and CCC				
10% sucrose	1.00 ± 0.07 d	3.24 ± 0.10 a	0.307 ± 0.019 a	0.307 ± 0.011 c
10% sucrose + BA 1.0	1.15 ± 0.07 cd	3.03 ± 0.06 b	0.273 ± 0.004 b	0.314 ± 0.012 bc
10% sucrose + BA 3.0	1.47 ± 0.06 bc	2.91 ± 0.05 b	0.243 ± 0.005 c	0.357 ± 0.059 bc
10% sucrose + BA 5.0	1.56 ± 0.05 bc	2.68 ± 0.09 c	0.239 ± 0.007 c	0.373 ± 0.017 ab
10% sucrose + BA 10.0	1.60 ± 0.11 bc	2.43 ± 0.11 d	0.231 ± 0.008 c	0.370 ± 0.012 ab
3% sucrose + BA 5.0 + CCC 500	2.26 ± 0.14 a	1.32 ± 0.07 e	0.067 ± 0.005 d	0.151 ± 0.010 d
10% sucrose + BA 5.0 + CCC 500	1.75 ± 0.09 b	2.54 ± 0.12 cd	0.242 ± 0.009 c	0.424 ± 0.019 a
Temperature (°C)				
10	0.00 c	0.00 d	0.000 d	0.000 c
15	1.89 ± 0.16 a	2.31 ± 0.13 c	0.187 ± 0.006 c	0.353 ± 0.022 b
20	1.63 ± 0.15 a	2.72 ± 0.15 b	0.268 ± 0.009 b	0.437 ± 0.018 a
25	1.25 ± 0.18 b	3.23 ± 0.43 a	0.304 ± 0.008 a	0.380 ± 0.014 b

Values represent means ± standard deviation. Means within a column followed by the same letter are not significantly different by Duncan's multiple range test (DMRT) at 0.05 probability level.

Temperature also shows significant effects on the rhizome formation. The number of rhizome decreased with the incubation temperature enhancement, but the length and fresh weight of rhizome increased from 2.31 ± 0.13 to 3.23 ± 0.43 cm and from 0.187 ± 0.006 to 0.304 ± 0.008 g, respectively (Table 4). At 20 °C, we got the highest fresh weight of rhizome per plantlet.

This paper, for the first time, presents the optimal culture conditions for the *in vitro* regeneration and the rhizome induction of manyflower betony using leaf explants,. These results provide a basis for germplasm *in vitro* conservation as well as rapid propagation of this species. *In vitro* microrhizome induction act as bioreactors, a promising approach to produce metabolites of interest, such as stachyose.

Previous studies on plant regeneration have shown that the response to types and concentrations of exogenous plant growth regulators was significantly different among various species of Stachys (Khussein *et al.* 2000, Li *et al.* 2002, Legkobit and Khadeeva 2004). In our study, although the results clearly showed that adventitious buds could regenerate on MS medium supplemented with either BA or TDZ in combination with NAA, BA proved to be more effective enhancer for bud multiplication and subsequent formation of normal shoots. Cheng *et al.* (2000) and Tang *et al.* (2002) also found that BA was more effective than TDZ in promoting shoot formation in cherry and betula platyphylla and the study of (Tzitzikas *et al.* 2004) found that TDZ suppresses the shoot growth in pea. The same phenomenon is observed in our study that only very few elongated shoots were obtained from the low concentration of TDZ treatment and these shoots looked abnormal, although some other studies exhibited that TDZ can induce higher bud regeneration rates than purine-based cytokinins in a number of species (Boufleuher *et al.* 2008, Corredoira *et al.* 2008).

High rates of shoot proliferation always are desirable for efficient micropropagation. For shoot organogenesis the subculture medium containing BA and NAA was essential. These hormones could stimulate bud regeneration and multiplication. In our study, these adventitious buds could be subcultured on this medium every 2 weeks for further buds proliferation. However, shoot buds always appeared as small clusters and only few shoots developed into normal plants. There have been reports of problems with poor elongation of induced buds in lingonberry, which may be due to the high cytokinin activity in medium (Lu 1993, Murthy *et al.* 1998, Debnath 2005). It is known that GA_3 can stimulate cell growth (Kotsias and Roussos 2001, Suzuki *et al.* 2004). On medium containing BA and NAA, our results were consistent with previous reports in which GA_3 was used successfully to elongate the induced shoot buds during the proliferation stage (Franklin *et al.* 2004).

The efficient rooting of regenerated shoots and the survival of plantlets in the soil are the important final steps for successful micropropagation. In this study, micropropagated shoots of manyflower betony rooted when cultured on MS auxin-free medium, but the maximum number of roots and faster root emergence were exhibited on medium with auxin. Both NAA and IAA achieved comparable rates of rooting *in vitro*, but NAA led to higher levels of shoot development, compared with IAA. The preference of a species to a specific auxin has been reported (Omura and Hidaka 1992, Goh *et al.* 1995). The superior effect of NAA on both root and shoot development has been previously documented in other plant species (Bergmann and Whetten 1998).

Our study is in agreement with previous reports that *in vitro* rhizome formation is highly dependent on sucrose concentration (Xu *et al.* 1998). MS medium supplemented with lower concentration sucrose (2%–5%) are recalcitrant to produce rhizomes. Higher levels of sucrose are also a requirement for microtuber formation in potato as reported by (Wang and Hu 1982) and (Khuri and Moorby 1995). Sucrose possibly acts as an energy source or a signal for rhizome induction. However, lower sucrose (3%) combination with 5.0 mg/L BA and 500 mg/L CCC produced the maximum number of rhizomes (Table 4). This indicated that CCC and BA can compensate the deficiency of sucrose and may be explained by the presence of CCC, which can reduce GA_3 biosynthesis and increases tuberonic acid synthesis, stimulating the rhizome formation (Stecco and Tizio 1982, Tovar *et al.* 1985, Vecchio *et al.* 1994).

Complete darkness was crucial during rhizome induction,and this has been observed in many other plant species (Hussain *et al.* 2006). Illumination changes the pathway of rhizome formation, and had a significant effect on the morphogenesis. (Dobranszki *et al.* 1999) and (Donnelly *et al.* 2003) demonstrated that microtuberization efficiency has been increased by short-day exposure or continuous darkness during culture condition. Light can stimulate GA_3 synthesis which inhibits rhizome induction while darkness enhanced tuberonic acid synthesis, which promotes rhizome formation (Alisdair and Willmitzer 2001, Jackson 1999, Simko *et al.* 1996).

REFERENCES

Alisdair R F and Willmitzer L. 2001. Molecular and biochemical triggers of potato tuber development. *Plant Physiology* **127**: 1 459–65.

Bergmann B A and Whetten R. 1998. *In vitro* rooting and early greenhouse growth of micropropagated *Paulownia* elongata shoots. *New Forests* **15**(2): 127–38.

Boufleuher L M, Schuelter A R, Luz C L, da Luz C L, Antes V A, Stefanello S, Comerlato A P and Otoni W C. 2008. *In vitro* propagation of *Solanum sessiliflorum* as affected by auxin and cytokinin combinations and concentrations. *Asian Journal of Plant Sciences* **7**(7): 639–46.

Cheng Z M, Schnurr J P and Dai W H 2000. Micropropagation of *Betula platyphylla* 'Fargo' via shoot tip culture and regeneration from leaf tissues. *Journal of Environ Hortic* **18**(2): 119–22.

Conkerton E J, Parrish F W, Chapital D C and Ory R L. 2006. Isolation of a stachyose-sucrose complex from soybeans and

peanuts. *Journal of Food Science* **48**: 1 269–71.

Corredoira E, Ballester A and Vieitez A M. 2008. Thidiazuron-induced high-frequency plant regeneration from leaf explants of *Paulownia tomentosa* mature trees. *Plant Cell Tissue and Organ Culture* **95**: 197–208.

Debnath S C. 2005. A two-step procedure for adventitious shoot regeneration from in vitro-derived lingonberry leaves: shoot induction with TDZ and shoot elongation using zeatin. *HortSci* **40**: 189–92.

Dobranszki J, Tabori K M and Ferenczy A. 1999. Light and genotype effects in vitro tuberization of potato plantlets. *Potato Research* **42**: 483–8.

Donnelly J D, Coleman W K and Coleman S E. 2003. Potato microtuber production and performance: a review. *American Journal of Potato Research* **80**: 103–15.

Franklin G, Carpenter L, Davis E, Reddy C S, Al-Abed D, Abou Alaiwi W, Parani M, Smith B, Goldman S L and Sairam R V. 2004. Factors influencing regeneration of soybean from mature and immature cotyledons. *Journal of Plant Growth Regulators* **43**: 73–9.

Goh C J, Sim G E, Morales C L and Loh C S. 1995. Plantlet regeneration through different morphogenic pathways in pommelo tissue culture. *Plant Cell Tissue and Organ Culture* **43**(3): 301–3.

Hayashi K, Nagamatsu T, Ito M, Hattori T and Suzuki Y. 1994. Acteoside, a component of *Stachys sieboldii* MIQ, may be a promising antinephritic agent (2): Effect of acteoside on leukocyte accumulation in the glomeruli of nephritic rats. *Japanese Journal of Pharmacology* **66**: 47–52.

Huang R L, Zhang Z H, Chen P G, Ma K W and Yang L. 2006. Effect of soybean-oligosaccharides on antioxidation and the immunity of broilers. *J Agr Univ HeBei* **29**: 88–94 (in Chinese).

Hussain I, Chaudhry Z, Muhammad A, Asghar R, Naqvi S M S and Rashid H. 2006. Effect of chlorocholine chloride, sucrose and ABP on *in vitro* tuberization in potato (*Solanum tuberosum* L. cv. cardinal). *Pakistan Journal of Botany* **38**(2): 275–82.

Jackson S D. 1999. Multiple signaling pathways control tuber induction in potato. *Plant Physiology* **119**: 1–8.

Khuri S and Moorby J. 1995. Investigations into the role of sucrose in potato cv. Estima microtubers production *in vitro*. *Annals of Botany* **75**: 295–303.

Khussein I A, Kochneva E Z and Khadeeva N V. 2000. Change in the spectra of peroxidases in regenerants of *Stachys sieboldii* (Miq.) as a result of hormonal and mutagenic effects. *Genetika* **36**(8): 1 093–9.

Kotsias D and Roussos P A. 2001. An investigation on the effect of different plant growth regulating compounds in *in vitro* shoot tip and node culture of lemon seedlings. *Scientia Horticulturae* **83**: 115–28.

Legkobit M P and Khadeeva N V. 2004. Variation and morphogenetic characteristics of different stachys species during microclonal propagation. *Russian Journal of Genetics* **40**(7): 743–50.

Li W, Gao H H, Lu R, Guo G Q and Zheng G C. 2002. Direct plantlet regeneration from the tuber of *Stachys sieboldii*. *Plant Cell Tissue and Organ Culture* **71**: 259–62.

Lu CY. 1993. The use of TDZ in tissue culture. *In Vitro Cell Dev Biol Plant* **29**: 92–6.

Murashige T and Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Plant Physiology* **15**: 473–97.

Murthy B N S, Murch S J and Saxena P K. 1998. Thidiazuron: a potent regulator of in vitro plant morphogenesis. *In Vitro Cell Dev Biol Plant* **34**: 267–75.

Nakakuki T. 2002. Present status and future of functional oligosaccharide development in Japan. *Pure and Applied Chemistry* **74**: 1245–51.

Omura M and Hidaka T. 1992. Shoot tip culture of Citrus. I. Longetivity of cultured shoots. *Bulletin of Fruit Tree Research Station* **22**: 37–47.

Simko I, Omer E A, Ewing E E, Mcmarry S and ko davis P J. 1996. Tuberonic (12-OH-jasmonate) acid glucoside and its methyl ester in potato. *Phytochemistry* **43**: 727–30.

de VL S and Tizio R. 1982. Action du CCC sur la tuberization des Germes de pomme de terres cultuves in vitro dans un milieu mineral depourvu de sucre. *CR Acad Sci Paris t. Serie III,* **294**: 901–3.

Suzuki P M, Kerbauy G B and Zaffari G R. 2004. Endogenous hormonal levels and growth of dark-incubated shoots of *Catasetum fimbriatum*. *Journal of Plant Physiology* **161**: 929–35.

Tang H R, Ren Z L, Reustle G and Krczal G. 2002. Plant regeneration from leaves of sweet and sour cherry cultivars. *Scientia Horticulturae* **93**(3–4): 235–44.

Tovar P, Estrada R, Schilde-Rentschler L and Dodds J H. 1985. Induction and use of *in vitro* potato tubers. CIP Circular 13, International Potato Centre, Lima, Peru, pp 1–5.

Tzitzikas E N, Bergervoet M, Raemakers K, Vincken J P, Lammeren AV and Visser RG F. 2004. Regeneration of pea (*Pisum sativum* L.) by a cyclic organogenic system. *Plant Cell Rep* **23**: 453–60.

Vecchio V, Ferraro S G, Pagano M T and Andrenelli L. 1994. Effect of saccaharose and CCC [(2-chloroethyl) trimethylammonim chloride] on in vitro production of microtubers of potato cultivars (*Solanum tuberosom*). *Sementi-Elette* **40**: 63–8.

Wang P and Hu C. 1982. In vitro tuberization and virus free seed-potato production in Taiwan. *American Potato Journal* **59**: 33–7.

Xu X, van Lammeren A A, Vermeer E and Vreugdenhil D. 1998. The role of gibberellin, abscisic acid, and sucrose in the regulation of potato tuber formation in vitro. *Plant Physiology* **117**: 575–84.

Zhang G Y, Li J and Cheng Z H. 2003. The cultivation of *Stachys floridana* Schuttl.ex Benth. *China Veg* **6**: 57–8 (in Chinese).

Zhao Y J and Yang Y H. 2007. Random double blind test on effect of soybean oligosaccharides in regulation of blood lipid in patients with hyperlipidemia. *Prog Mod Biomed* **7**: 1526–35 (in Chinese).

Zhong X F, Huang G D, Li S B and Xu BC. 2006a. Study on extraction of polysaccharidesfrom *Stachys floridana* Schuttl.ex Benth. *Acad Period Farm Prod Process* **11**: 39–41 (in Chinese).

Zhong X F, Huang G D, Yi J P and Xu B C. 2006b. The development and utilization of the manyflower betony. *Food and Nutrition in China* **8**: 20–2.