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Buds Induction and High-frequency Plant Regeneration of *Salivia miltiorrhiza* Bunge

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Abstract: A direct induction of adventitious buds and *in vitro* plantlet regeneration system for *Salivia miltiorrhiza* Bunge was optimized by studying the influences of genotype, explants type (young stem, petiole and leaf) and different concentration of BA, IBA and surcose. Leaf explants from 99-5 seedlings showed maximum buds induction. The plant growth regulator BA (0.1 mg · L^{-1}) was effective in stimulating shoot regeneration from leaf explants of *S. miltiorrhiza*. The highest efficiency of bud formation was observed with a 30-day culture in MS containing 1.0 mg · L^{-1} BA. The regenerated buds were transferred to MS medium containing 0.1 mg · L^{-1} BA for elongation. When the shoots were about 3.5 cm in height, they were transferred to 1/2-strengh MS medium supplemented with 1.0 mg · L^{-1} IBA and 10% sucrose to induce rooting.

Key words: *Salivia miltiorrhiza* Bunge; adventitious bud; leaf explant; regeneration

丹参丛生芽诱导和植株的高频再生

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摘　要：对丹参直接芽再生系统进行研究，探讨不同基因型、外植体类型(幼茎，下胚轴和叶)和 BA、IBA 及蔗糖的浓度对其不定芽诱导、伸长和生根的影响。结果表明，来自丹参 99-5 幼苗的叶外植体芽诱导率最大。诱导芽再生的最佳培养基为 MS＋0.1mg · L^{-1}BA，在该培养基上培养 30 d 的外植体可获得最多的不定芽。将再生芽转移到 MS＋0.1mg · L^{-1}BA 培养基上进行伸长培养，当芽长至 3.5cm 时，将其转移至 1/2 MS＋1.0 mg · L^{-1} IBA＋10% 蔗糖的培养基中诱导生根。

关键词：丹参；不定芽；叶外植体；再生
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The Radix of *Salvia miltiorrhiza* Bunge (Labiatae), an eminent medicinal plant, is a traditional Chinese medicine. Some diterpenoid quinine compounds, such as tanshinone IA, tanshinon IIA, cryptotanshinone and isocryptotanshinone are effective composition. The dried root of *Salvia miltiorrhiza*, for example, has been used extensively for the treatment of coronary and cerebrovascular disease, sleep disorders, HIV, hepatitis, hepatocirrhosis, chronic renal failure, dysmenorrhea, amenorrhea, carbuncles and ulcers, and it is often included as an ingredient in various herbal remedies recommended for vascular circulation therapies[1~5]. It showed that the prospect of developing new Radix medicines would have curative effect to a great number of serious diseases. This is cheerful. But low content of effective components in Radix and its long growing period make it difficult to control the clinical quality[6]. Thus, it may be possible to solve these problems by biotechnique of *S. miltiorrhiza* to improve quality and yield, obtain effective components from cultured cells and organs of *S. miltiorrhiza et al.* The first step to achieve these goals is to establish an efficient *in vitro* system with high regeneration frequency for *S. miltiorrhiza*. There are some

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reports of *in vitro* proliferation of bud induced by plant hormone. Tian *et al.* have recently reported the high potential of leaf for callus induction[7], but direct regeneration has not been reported yet. We present an *in vitro* regeneration system based on leaf explants obtained from adult plants of *S. miltiorrhiza*. The results of these experiments provide for the first time a standardized protocol for high frequency plant regeneration through direct organogenesis in *S. miltiorrhiza*.

1 MATERIALS AND METHODS

1.1 Plant materials and explants preparation

Radix *Salivia miltiorrhiza* Bunge seeds were obtained from Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, and were grown in the field or in the greenhouse. Three genotypes, namely DS2-2000, B0-1 and 99-5 were used in the experiments. Seeds were surface-sterilized by immersion in 70% (v/v) ethanol for 30 s, followed by treatment with 1% (w/v) $HgCl_2$ for 15 min and washed 3 ~ 4 times with sterile distilled water. Seeds were then germinated on one-half strength hormone-free MS medium. Young stem (5 ~ 8 mm)、petiole (5 ~ 8 mm) and leaf (20 ~ 25 mm^2) explants were excised from one-month-old seedlings.

1.2 Buds differentiation

Bud regeneration was carried out on MS basal medium supplemented with 30 g · L^{-1} sucrose and 0.7% agar. Five concentration of BA (0.1, 0.5, 1.0, 2.0, 3.0 and 5.0 mg · L^{-1}) were tested for callus induction and its subsequent growth from various explants. Medium lacking growth regulator was used as control. All *in vitro* cultures were incubated at 25 ± 1℃ under a 16 h photoperiod (light intensity is 27 μmol · m^{-2} · s^{-1}).

1.3 Shoot growth and rooting

Induced buds were transferred after 20 d in the same type of culture medium for growth and shoot regeneration. Individual shoot was subcultured on MS medium supplied with 0.1 mg · L^{-1} BA for elongation and multiplication. After the regenerated shoots had reached a height of 3 ~ 4 cm, they were individually excised from clumps and transferred to glass triangle bottles (GenTel, Beijing) containing 30 mL 1/2-strength MS medium with IBA (0.01, 0.1, 0.5, 1.0 and 2.0 mg · L^{-1}, respectively) and sucrose (10, 20 and 30 g · L^{-1}, respectively) for rooting. Rooted plantlets were transferred to plastic pots containing autoclaved vermiculite-soil (1:1, v/v). The pots were covered with plastic film, placed in a greenhouse and irrigated with a solution of 1/10-strength MS inorganic salts at 3 d intervals.

1.4 Data analysis

Each experiment was repeated three times with at least 20 explants per treatment. The percentage of young stem, petiole and leaf explants producing callus (SPC, PPC and LPC) was determined after 30 d of growth. Regenerated shoots (2.5 ~ 3.5 cm long) were transferred to rooting medium. The average number of rooted shoots was calculated that had been transferred on the rooting medium for one week. The data were analyzed using SAS (6.12). Analysis of variance (ANOVA) was used to test the statistical significance, and the significance of differences among means was carried out using Duncan's multiple-range test at $P = 0.05$.

2 RESULTS

2.1 Effect of explant types

The responses of various explants [young stem (SE), petiole (PE) and leaf (LE)] regarding callus induction and growth are presented in Tables 1 and 2. Callus mass was initiated with 25 ~ 30 d directly on the cut surfaces in all three types of explants on MS basal medium supplemented with various concentration of BA. However, callusing rate was significantly affected by the type of primary explants used in the following order: LE > PE > SE. Leaves were the best source of explants for buds regeneration (81%). Over a period of 50 d, 92% of leaf-derived callus produced shoots, with a highest average of 35 shoots per explants.

2.2 Effect of the concentration of BA on buds induction

Buds initiation was observed from the cut ends of the young stem, petiole and leaf explants within 25 ~ 30 d of incubation, while no bud was observed on control media.

The data for buds induction frequency, in terms of the percentage of explants that showed buds formation, was calculated for each explants type subjected to individual phyto-hormone concentration for all the genotypes studied.

Among the various concentrations of BA studied, 1.0 mg · L^{-1} was observed to be highly potent for buds induction irrespective of explants and genotypes studied with pooled average buds induction frequency of 49.8% based on a total of 2 700 explants (20 explants, 3 genotypes, 3 explants types, 5 concentrations, in triplicate). The leaf was observed to be the least responsive to buds induction, the highest pooled buds induction frequency being 81.2% on 1.0 mg · L^{-1} BA (Table 1). No remarkable difference was observed in buds induction frequency between different genotypes. The pooled average buds induction frequency was 24.2% for DS2-2000, 24.1% for B0-1 and 25.4% for 99-5, respectively, based on a total of 900 explants (20 explants, 3 explants types, 5 concentrations, in triplicate). Buds induction was effected remarkably by explants type. The pooled average buds induction frequency of leaf (38.55%) was remarkably higher than stem (16.7%) and petiole explants (18.36%). And remarkable difference was discovered between different concentrations of BA (Table 2). The shoots obtained on media containing 1.0 mg · L^{-1} BA showed normal leaf development and rapid stem elongation on further subculture on MS medium supplemented with 0.1 mg · L^{-1} BA.

Table 1 Effect of BA concentration on the percentage of *S. miltiorrhiza* producing buds.

Concentration of BA (mg · L^{-1})	Buds induction and growth (%)								
	DS2-2000			B0-1			99-5		
	SPS	PPS	LPS	LPS	SPS	PPS	SPS	PPS	LPS
Control	0	0	0	0	0	0	0	0	0
0.1	5.8	9.6	11.2	5.3	7.5	9.8	6.8	8.9	10.5
0.5	17.6	19.4	31.2	18.5	16.4	29.5	21.5	24.3	30.2
1.0	34.2	33.1	75.4	35.6	37.2	79.5	35.6	36.5	81.2
2.0	25.7	31.2	65.5	27.1	27.8	67.2	28.1	31.2	69.5
3.0	11.2	13.5	32.1	10.5	12.1	29.8	9.8	13.2	27.6
5.0	2.5	3.1	12.5	2.1	2.8	14.7	2.6	2.7	16.5

Notes: Values represented the means calculated from percentage of 20 explants forming buds in triplicate for growing 30 d. MS was used as a control.

Table 2 The average induction frequencies of three factors and their significance differences.

Genotype	Mean	Explants	Mean	Concentration of BA(mg · L^{-1})	Mean
DS2-2000	24.2a	stem	16.70b	0.1	8.4c
B0-1	24.1a	petiole	18.36b	0.5	23.2b
99-5	25.4a	leaf	38.55a	1.0	49.8a
				2.0	41.5a
				3.0	17.8b
				5.0	6.6c

Notes: The means indicated induction frequencies, which were caculated from 20 explants in triplicate. The different letters within the columns showed significantly different at 5% level of significance according to Duncan's multiple range tests.

2.3 Influence of concentration of IBA and sucrose on root formation

After reaching a height of 2.5 ~ 3.5 cm, the shoots were seperated from the clump and transferred to 1/2-strength MS medium supplemented with various concentrations of IBA and sucrose produced roots at varying efficiencies (Table 3). Adventitious shoots grew quickly following their transfer to rooting medium. The best result was obtained on 1/2-strength MS medium containing low concentration of 0.1 mg · L^{-1} IBA and 1.0% sucrose for rooting. About 90% of the shoots rooted successfully (Fig. 1). Rooted plantlets were transplanted into plastic pots containing autoclaved vermiculite and soil (1∶1, v/v) for further growth. After 3 weeks, the surviving plants were transplanted into the field.

Table 3 Effect of IBA and sucrose concentration on shoots rooting.

Concentration of IBA (mg · L^{-1})	Concentration of sucrose (g · L^{-1})		
	10	20	30
0.01	83.6b	76.4b	75.7b
0.1	95.3a	89.0a	84.3a
0.5	68.4c	61.2c	61.8c
1.0	53.3d	51.5d	46.6d
2.0	32.4e	22.9e	19.2e

Note: Data were means of triplicates.

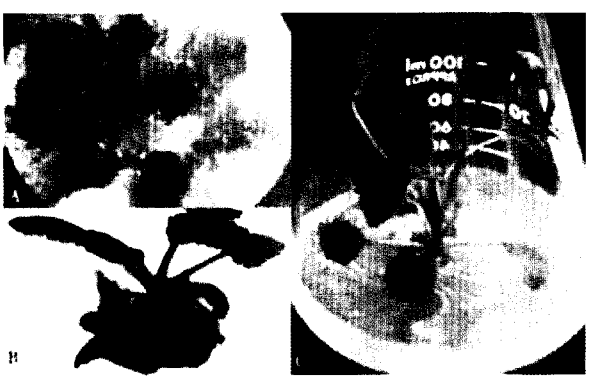

Fig. 1 Direct regeneration of *S. miltiorrhiza*.

A. Adventitious buds formation from leaf explants of *S. miltiorrhiza* in MS containing 1.0 mg · L^{-1} BA. Adventitious shoot regenerated at the margins of leaf explants after a 40 d culture. B. Elongated shoots that had developed from leaf explants on MS medium containing 1.0 mg · L^{-1} BA after a 15 d culture. C. Rooted plantlet on 1/2-strength MS medium containing 0.1 mg · L^{-1} IBA and 10% sucrose.

3 DISCUSSION

We have developed a highly efficient system for shoot direct regeneration from *S. miltiorrhiza* leaf tissue. The best treatment was leaves from seedlings cultured on MS medium and shoots formation can be induced from *S. miltiorrhiza* leaf explants using MS medium containing 1.0 mg · L^{-1} BA. In the majority of the experiments, 80% explants produced shoots.

The results of the present investigation reflect the existence of large inter-explant variability in budding responses of the target taxa. Such variable responses for different explant types have also been reported in other species [7~9]. Such variations can be attributed to the physiological condition of the explants, which is determined by genetic factors[10, 11]. The regeneration capacity of leaf explant is higher than stem and petiole explants in *S. miltiorrhiza*. According to Hosoki and Asahira, intercalary meristems distributed in leaves might be responsible for the higher regeneration potential [12].

In this study, 1.0 mg · L^{-1} BA was found to be the best concentration for induction of adventitious shoot from *S. miltiorrhiza* leaves. Cytokinin-auxin combination has been widely used for callus regeneration in various protocols developed for other members of the Labiatae[13~16]. But studies on *S. miltiorrhiza* indicate good responses in media supplemented with single cytokinin. The method revealed some advantages in comparison with others: A large number of buds regenerate from each explant. Buds regeneration are simultaneous in the same medium, but in shorter time (only 30 ~ 50 d). There are no subculturing and no undesirable visibly variations.

Root formation was effected by concentration of sucrose. Frequency of root formation was the highest in media contained 10 g · L^{-1} sucrose. The variable rooting responses for different concentration of sucrose have also been reported in other plants[17~21]. The results can be attributed to the regulation of metabolic and osmotic potential. Sucrose in media supplies energy substrate for root

growth, on the other hand, it affect the osmotic potential of media. Both metabolic regulation and osmotic potential regulation participate in the root formation.

In general, the present investigation reports an efficient and easy-to-handle protocol for organogenesis. Leaf is the best explant source for the species and 80% callus can be induced in MS medium supplemented with 1.0 mg · L^{-1} BA. Furthermore, 90% callus differentiation along with a multiplication rate of 25 shoots per explant can be obtained in 1.0 mg · L^{-1} BA, and 95% of these shoots can be rooted in one-half strength MS medium supplemented with 0.1 mg · L^{-1} IBA and 10 g · L^{-1} sucrose. Following the procedures described here, approximately 20 well-rooted plantlets can be developed from a single explant. This protocol could be very useful in multiplying elite of the target species with limited time duration. The importance of developing callus lines has increased over the years because of active compound production, and the possibility of genetic transformation. The present callus regeneration system may also be important for advanced studies on genetic improvement and in the future, it also has considerable potential as an alternative means for production of known and new secondary metabolites and pharmaceutical proteins.

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Reference

[1] XU R S H. Radix *S. miltiorrhiza*—biology and application [M]. Beijing: Science Press, 1990,158(in Chinese).

[2] Ryu S Y, Lee C O, Choi S U. *In vitro* cytotoxicity of tanshinones from *S. miltiorrhiza* [J]. Planta Med., 1997, 63 (4): 339-342.

[3] Zheng M S H, Qiu J H. Experimental investigation into assessment of antiviral herbs against HBeAg using ELISA technique [J]. Natural Product Research and Development, 1995, 7 (1):10-15.

[4] Zhu L J. Research progress of anti-HIV Chinese herbal medicine and prescription [J]. Chinese Journal of Information on TCM, 1996, 3: 17(in Chinese).

[5] Huang Y S, Zhang J T. Antioxidative effect of three water-soluble components isolated from *Salvia miltiorrhiz in vitro* [J]. Acta Pharmaceutica Sinica, 1992, 27(2): 96(in Chinese).

[6] Yuan L, Su G L, Hu G S. On the quality of danshen injection and compound danshen injection [J]. Chinese Traditional and Herbal Drugs, 1994, 25(6): 299-303(in Chinese).

[7] Christopher T, Rajam M V. Effect of genotype, explant and medium on *in vitro* regeneration of red pepper [J]. Plant Cell Tissue Organ Cult., 1996, 46: 245-250.

[8] Arockiasamy S, Ignacimuthu S. Plant regeneration from mature leaves and roots of *Eryngium foetidum* L., a food flavouring agent [J]. Curr. Sci., 1998, 75: 644-666.

[9] Pereira A M S, Bertoni B W. Micrpopropagation of *Pathomorphe umbellate* via direct organogenesis from leaf explants [J]. Plant Cell Tissue Organ Cult., 2000, 60: 47-53.

[10] Baroncelli S, Buitti S. Genetic control of *in vitro* and *in vivo* growth in hexaploid wheat [J]. Z. Pflanzenzuecht, 1978, 80: 109-116.

[11] Nagarathna K C, Prakash H S, Shetty H S. Genotypic effects on the callus formation from different explants of pearl millet B lines [J]. Adv. Plant Sci., 1991, 4: 82-86.

[12] Hosoki T, Asahira T. *In vitro* propagation of bromeliads in liquid culture [J]. Hortic. Sci., 1980, 15: 603-604.

[13] Liu M, Tian W, Jiao Y G, *et al.*. Study on tissue culture for mint [J]. Research and Practice of Chinese Medicines, 2005, 19(2): 8-11(in Chinese).

[14] Cai H Q. Tissue culture and rapid propagation of *Ocimum basilicum* L [J]. Plant Physiology Letter, 2005, 41(3):347.

[15] Li F L, Hu G F, Hu B Z. Study on tissue culture and plant regeneration of the inflorescence in eight kinds color of *Saliva splendens* [J]. Biotechnology, 2005, 15(4): 71-73.

[16] Tian Y H, Wang Z Z. Tissue culture and plantlet regeneration of *Salivia miltiorrhiza* Bunge [J]. Journal of Shanxi Normal University (Natural Science Edition), 2003, 31(1): 99-102 (in Chinese).

[17] Zhang W J, Wang T. Construction the system of high frequency regeneration from callus of alfalfa and study on the effect factors [J]. Scientia Agricultura Sinica, 2002, 35(12): 1579-1583(in Chinese).

[18] Li T Z, Li B T, Wang X R. Effect of sugar and N to tip rooting of elm tissue culture [J]. Shanxi Scientific and Technical Forestry, 2001, 4:11-13(in Chinese).

[19] He X J, Liu S H, Feng Y L, *et al.*. The study on rapid tissue culture propagation technology of *Dracaena cambodinna* [J]. Guangdong Scientific and Technical Forestry, 2006, 22(1): 22-25(in Chinese).

[20] Guo W Z, Lin Y, Bi J, *et al.*. Study on tissue culture of *Zanthoxylum piperitum* DC var. inerme makino [J]. China Forestry Science and Technology, 2005, 19(5): 19-21(in Chinese).

[21] Zuo Y Z, Cui B I, Li C X, *et al.*. Primary study on mutant *Populus tomentosa* in tissue culture [J]. Heibei Journal of Forestry and Orchard Research, 2006, 21(1): 10-13(in Chinese).