Improvement of Growth and Periplocin Yield of *Periploca sepium* Adventitious Root Cultures by Altering Nitrogen Source Supply

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Abstract: **Objective** To increase the ultimate yield of periplocin in *Periploca sepium* adventitious root cultures by a two-stage culture based on nitrogen source. **Methods** Firstly, the effects of nitrogen source (NH_4^+-NO_3^-) at different ratios and different total initial nitrogen amounts on the accumulation of biomass and secondary metabolites in adventitious root cultures of *P. sepium* were investigated, and growth and production media for the two-stage culture based on the above results were established. **Results** The highest biomass and periplocin content were obtained in the culture medium of 15 mmol/L total nitrogen amount with NH_4^+-NO_3^- (1:2) and 30 mmol/L total nitrogen amount with nitrate as the sole nitrogen source. By adopting a fed-batch cultivation strategy, the dry weight adventitious root, periplocin content and yield were increased by 136%, 108%, and 389%, respectively when compared with those of the control, reaching up to 8.13 g/L, 157.15 μg/g, and 1277.63 μg/L, respectively. Furthermore, it was found that in the process of two-stage culture, the adventitious roots grew thicker significantly after they were transferred into production medium directly. **Conclusion** The ultimate yield of periplocin in *P. sepium* adventitious root cultures could be significantly increased by a two-stage culture based on nitrogen source.

Key words: adventitious root; nitrogen source; *Periploca sepium*; periplocin yield; two-stage culture

DOI: 10.3969/j.issn.1674-6384.2011.03.010

Introduction

The root bark of *Periploca sepium* Bunge is also known as northern Wu Chia Pee (*Periplocae Coxtex*) which has been used for over hundreds of years as a traditional medicinal plant. Different from Wu Chia Pee, *P. sepium* has toxicity to the body instead of nutritions (Wang *et al*, 2010; Chen *et al*, 2005). The origin of its toxicity comes from periplocin (Zhou *et al*, 2011), one of the most important active ingredients, which has been proven to have cardiotonic and antitumor effect (Kit, 1964; Umehara *et al*, 1995; Liu *et al*, 2004; Zhang *et al*, 2010). Modern pharmacological researches have shown that the cardiotonic effect of periplocin has some characteristics, such as high-speed, short-duration, and non-cumulation (Wang *et al*, 2008). In the study of *in vitro* experiments, Wang *et al* (2010) found periplocin could increase cell viability to a level lower than ouabain in the MTT analysis, but decrease LDH release simultaneously, which means periplocin has lower cytotoxicity compared to ouabain, and thus can provide a new insight into the treatment of heart failure. Now its root bark, *Periplocae Coxtex*, as a traditional Chinese medicinal msterial, is chiefly used to treat cardiac failure (Wang *et al*, 2010; Xu, Lu, and Zhang, 1998). For a long time, *P. sepium* has been obtained mainly from wild and tissue culture of this species has seldom been reported yet. So it is significant to acquire its active ingredient of periplocin by the mode of tissue

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Received: February 25, 2011; Revised: March 29, 2011; Accepted: June 14, 2011
Fund: Main Project of Tianjin Science and Technology Support Program (09ZCKFSH01100)

culture. Our laboratory successfully obtained the subculture system of *P. sepium* adventitious roots, which could provide a new way for increasing the medicinal herb resource and producing periplocin. The main objective of our experiment was to investigate the effect of nitrogen source ratio (NH_4^+/NO_3^-) and total initial nitrogen amount on *P. sepium* adventitious root growth and periplocin production, and on this basis to establish a two-stage culture system based on nitrogen source to obtain the maximum periplocin yield.

Materials and methods

Plant materials

Adventitious roots were induced from *in-vitro*-grown plantlets of *P. sepium* which had grown from sterilized mature seeds collected from the wild plants grown in Jixian mountainous area (Tianjin, China). The collected seeds were washed under running tap water for 40 min, then were further sterilized in surface with 75% alcohol for 30 s, followed by soaking in 4% NaOCl solution for 20 min, and rinsed with plenty of sterile distilled water. The sterilized seeds were clipped with a pair of sterilized forceps and inoculated in conical flasks containing 50 mL Murashige and Skoog (MS) basal medium supplemented with 3% sucrose and 0.8% agar. Cultures were maintained at (25 ± 2) ℃ under a 16/8 h (day/night) photoperiod to develop plantlets. These plantlets produced from this culture (after three weeks) were used as the source of root explants (1 cm long) for inducing adventitious roots.

Adventitious root induction and proliferation

The selected root explants were placed in solid 1/2 MS medium supplemented with 1 mg/L indole butyric acid (IBA), 3% sucrose, and 0.8% agar in Petri dishes containing 25 mL medium for adventitious root induction. Cultures were maintained under darkness at (23 ± 2) ℃ for four weeks. Then the induced adventitious roots were further proliferated in 100 mL 1/2 MS liquid medium on gyratory shakers at 120 r/min every 25 d. The culture condition of proliferation medium was the same as the induction medium.

Investigation on nitrogen source (NH_4^+/NO_3^-) at different ratio and different total initial nitrogen Amount

Adventitious roots (6 g/L, 1 cm long) were inoculated in 100 mL shake flasks containing 50 mL 1/2 MS medium supplemented with 1.0 mg/L IBA, 3% sucrose, and various NH_4^+-NO_3^- (0:30, 5:25, 10:20, 15:15, 20:10, 25:5, and 30:0, using NH_4Cl and KNO_3). The root growth and periplocin production were analyzed after 25-day culture in order to determine the optimum NH_4^+/NO_3^- ratio for cultures growth and periplocin production, respectively. Then adventitious roots were further investigated by using various total initial nitrogen amounts (15, 30, 45, 60, and 90 mmol/L) with a NH_4^+/NO_3^- ratio of 1:2 or nitrate as the sole nitrogen source, respectively. All shake flask cultures were kept in dark at (23 ± 2) ℃ and on gyratory shakers at 120 r/min. The root growth and periplocin production were analyzed after 25-day culture. Each experiment was repeated for at least three times.

Two-stage culture based on nitrogen source

Firstly, adventitious roots (6 g/L, 1 cm long) were cultured in growth medium [NH_4^+-NO_3^- (1:2, 15 mmol/L)] for 20 d and then the cultures (without being cut into segments) were transferred into production medium (nitrate as sole nitrogen, 30 mmol/L) directly for another 16 d. 1/2 MS basal culture medium [NH_4^+-NO_3^- (1:2, 30 mmol/L)] was treated as control. The cultures in experimental and control groups were sampled every four days in this process to determine the biomass and periplocin content in *P. sepium* adventitious root cultures. Each experiment was repeated for at least three times.

Determination of biomass

The adventitious roots were collected from the medium by using a pair of forceps, afterwards they were rinsed with tap water before oven drying. Dry weight was recorded after the adventitious roots were dried to constant weight at 50 ℃ for 24 h.

Determination of periplocin in adventitious roots

The dried root sample (0.05 g) was extracted once with 20 mL 50% ethanol for 40 min at 40 ℃. Following the filtration, the extract was evaporated to dryness and then dissolved in 2 mL 50% ethanol. The extract was then used for the identification and quantification of periplocin by HPLC. The periplocin fraction was analyzed using HPLC system (Agilent 1100, USA) on Hypersil ODS2 C_{18} (250 mm × 4.6 mm, 5 μm). Acetonitrile-methanol-water (25:10:65) was employed as eluent with flow rate of 1.0 mL/min. Sample (20 μL) was injected into HPLC and the peak was monitored at 220 nm (Ren *et al*, 2007). According to the regression

equation, $Y = 15\ 693\ X - 1884.3$, $r = 0.9993$, the content of periplocin was calculated.

Statistical analysis

The statistical analysis was performed according to V11.5 SPSS system. Mean and standard errors were used throughout and the statistical significance between the mean values was assessed applying a Duncan's multiple range tests. A probability of $P < 0.05$ was considered significantly.

Results

Effect of NH_4^+/NO_3^- on accumulation of biomass and periplocin in adventitious root

The effect of NH_4^+/NO_3^- on biomass and periplocin accumulation in *P. sepium* adventitious root was determined after 25-day culture, using a total initial nitrogen amount of 30 mmol/L (Table 1). In this study, nitrate was found to result in better root growth and higher periplocin accumulation. The optimum biomass of 3.57 g/L was obtained when the NH_4^+-NO_3^- was 1:2. The highest periplocin content (145.64 μg/g) was obtained when nitrate was the sole nitrogen source.

Effect of total initial nitrogen amount on accumulation of adventitious root biomass and periplocin

The effect of total nitrogen amount on *P. sepium* adventitious root biomass and periplocin accumulation was determined after 25-days culture, using a NH_4^+-NO_3^- of 1:2 (in favor of growth) and nitrate as sole nitrogen source (in favor of periplocin formation), respectively. As shown in Table 2, adventitious root growth and periplocin production were inhibited at high total initial nitrogen amount. The most favorable total nitrogen amount for maximum biomass (3.64 g/L) was 15 mmol/L with NH_4^+-NO_3^- of 1:2. The highest periplocin content (142.92 μg/g) was obtained at 30 mmol/L (nitrate as sole nitrogen source). In addition, periplocin accumulation in adventitious roots cultivated in the medium with 15 mmol/L nitrogen nutrient with NH_4^+-NO_3^- of 1:2 (growth medium) was much lower than that in those cultured ones in the medium with 30 mmol/L nitrate as the sole nitrogen source (production medium). Likewise, adventitious root growth in production medium was inferior to that in growth medium. Therefore, to optimize culture efficiency, adventitious root growth and secondary metabolism should be separated by using a two-stage culture.

Table 1 Effect of NH_4^+/NO_3^- ratio on growth and periplocin accumulation in adventitious root cultures ($\overline{X} \pm s$, 25 d)

NH_4^+/NO_3^-	Dry weight / ($g \cdot L^{-1}$)	Periplocin content / ($\mu g \cdot g^{-1}$)	Periplocin yield / ($\mu g \cdot L^{-1}$)
0:30	1.81 ± 0.15 c	145.64 ± 8.31 a	263.61 ± 17.23 a
5:25	2.83 ± 0.19 b	104.73 ± 7.24 b	296.39 ± 21.43 a
10:20	3.57 ± 0.22 a	76.96 ± 3.49 c	274.75 ± 18.09 a
15:15	3.12 ± 0.17 ab	59.51 ± 3.07 d	185.67 ± 12.54 b
20:10	2.76 ± 0.10 b	41.92 ± 1.75 e	115.70 ± 6.18 c
25: 5	1.55 ± 0.18 d	37.30 ± 1.62 e	57.82 ± 4.24 d
30: 0	1.02 ± 0.07 d	30.94 ± 2.84 e	31.56 ± 2.51 d

Adventitious root cultures were maintained in modified 1/2 MS liquid medium by altering NH_4^+/NO_3^- at 30 mmol/L total nitrogen amount

Letters mean separation within column by Duncan's multiple range tests at $P < 0.05$

a－e: significance at 0.05 level

Improvement of adventitious root growth and periplocin yield by two-stage culture

In the process of two-stage suspension culture, dry biomass and periplocin content and yield in adventitious root cultures increased by 136%, 108%, and 389%, respectively when compared with those of control, reaching up to 8.13 g/L, 157.15 μg/g, and 1277.63 μg/L, respectively (Table 3, Fig. 1A and 1B). Furthermore, in this study, it was found that in the process of two-stage culture, the adventitious roots grew obviously thicker after they were transferred from the growth medium to the production medium directly, which caused the roots to have more dry biomass (Figs. 1A and 2).

Discussion

Ammonium to nitrate ratio

It is a general trend that nitrate, compared with ammonium, was more favorable to the cultures growth or proliferation (Pan *et al*, 2004; Panda, Mishra, and Bisaria, 1992; Chen *et al*, 2003; Bensaddek *et al*, 2001; Cui *et al*, 2010; Huang *et al*, 2010). In this study, nitrate

Table 2 Effect of initial total nitrogen amount on growth and periplocin accumulation in adventitious root cultures ($\bar{X} \pm s$, 25 d)

Total nitrogen amount / (mmol·L^{-1})	Dry weight / (g·L^{-1})	Periplocin content / (μg·g^{-1})	Periplocin yield / (μg·L^{-1})
15[a]	3.64 ± 0.28 a	59.15 ± 4.73 e	215.31 ± 19.05 b
30[a]	3.51 ± 0.19 ab	78.02 ± 2.13 d	273.85 ± 8.78 a
45[a]	3.02 ± 0.11 b	54.27 ± 4.99 ef	163.90 ± 12.29 b
60[a]	2.69 ± 0.14 b	38.71 ± 2.60 fg	104.13 ± 7.64 cd
90[a]	2.16 ± 0.15 c	32.98 ± 1.87 g	71.24 ± 5.81 d
15[b]	2.08 ± 0.08 c	106.15 ± 8.04 bc	220.79 ± 14.68 b
30[b]	1.76 ± 0.15 cd	142.92 ± 8.46 a	251.54 ± 13.93 ab
45[b]	1.68 ± 0.04 cd	110.48 ± 4.91 b	185.61 ± 8.37 b
60[b]	1.44 ± 0.12 de	90.55 ± 7.34 cd	130.39 ± 12.91 bc
90[b]	1.18 ± 0.07 e	59.38 ± 4.26 e	70.07 ± 5.74 d

Adventitious root cultures were maintained in modified 1/2 MS liquid medium by altering initial nitrogen amount

Mean separation within column by Duncan's multiple range tests at $P < 0.05$

a: Nitrogen at NH_4^+-NO_3^- (1:2) as nitrogen source in 1/2 MS liquid medium and other components were not changed

b: Nitrate as sole nitrogen source in 1/2 MS liquid medium and other components were not changed

letters a—e: significance at 0.05 level

Table 3 Improved periplocin yield in adventitious root cultures by two-stage culture ($\bar{X} \pm s$)

Culture method	Dry weight / (g·L^{-1})	Periplocin content / (μg·g^{-1})	Periplocin yield / (μg·L^{-1})
Control (one-stage culture)	3.45 ± 0.22 (day 24)	75.68 ± 5.83 (day 24)	261.10 ± 19.64 (day 24)
Two-stage culture	8.13 ± 0.36 (day 32)	157.15 ± 12.95 (day 32)	1277.63 ± 109.24 (day 32)

Maximum periplocin yield was obtained at day 24 in process of control (one-stage culture)

Maximum periplocin yield was obtained at day 32 in process of two-stage culture

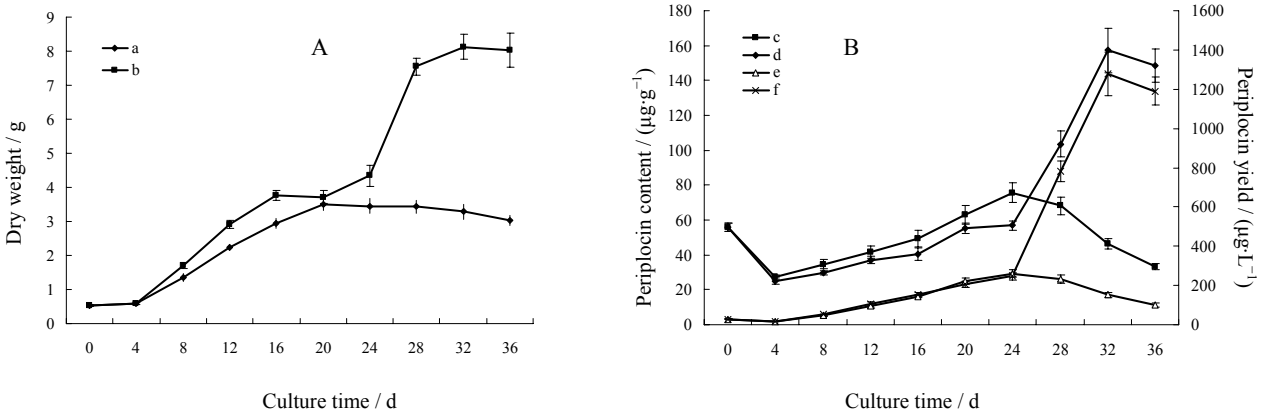

Fig. 1 Curves of growth (A) and periplocin accumulation (B) in adventitious root cultures in process of one-stage and two-stage culture

a: Dry weight in process of one-stage culture b: Dry weight in process of two-stage culture c: Periplocin content in process of one-stage culture d: Periplocin content in process of two-stage culture e: Periplocin yield in process of one-stage culture f: Periplocin yield in process of two-stage culture

was also favorable to *P. sepium* adventitious root growth. The possible reason is that ammonium is very diffusive and easily accumulates into the tissue, and becomes toxic if not immediately metabolized (Bensaddek *et al*, 2001). Another consequence of the accumulation of ammonium could be a restraint of nitrate assimilation (Crawford, 1995), which would result in medium acidification. Furthermore, the results indicated that the root growth was also inhibited significantly if the nitrate was acted as the sole nitrogen source. This was probably due to medium acidification caused by excessive nitrate, which was also deleterious to root growth.

In terms of accumulation of secondary metabolite, there are mainly two situations. In the first case, ammonium is favorable to accumulation of secondary metabolite, e.g. alkaloid (Moreno, Heijden, and Verpoorte, 1995; Pan *et al*, 2004; Panda, Mishra, and Bisaria, 1992; Chen *et al*, 2003; Bensaddek *et al*, 2001). In the second case, nitrate is favorable to accumulation of secondary metabolite (Wang and Tan, 2002; Cui *et al*, 2010; Zhang, Zhong, and Yu, 1996; Huang *et al*, 2010). In this study,

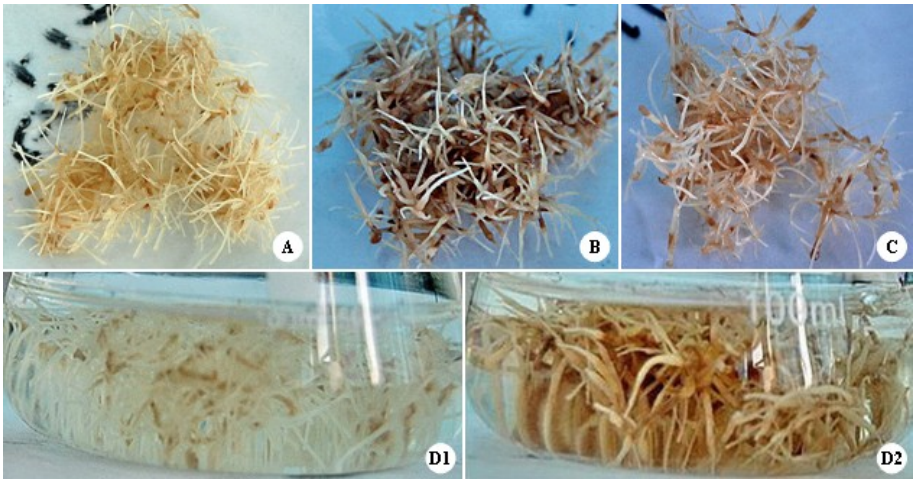

Fig. 2 Growth situation of adventitious roots in growth medium and production medium respectively

A: Adventitious roots at the first day 20 in growth medium B: Adventitious roots (without being cut into segments) at the next day 12 in production medium C: Adventitious roots (being cut into segments) at day 25 in production medium D: Contrast between the growth situation of adventitious root cultures in growth medium (D1) and that in production medium (D2) in the process of two-stage culture

nitrate is favorable to periplocin production in *P. sepium* adventitious root cultures. This phenomenon shows that the effect of nitrogen source (NH_4^+ and NO_3^-) on secondary metabolite formation is very complicated, which depends on both species and kinds of secondary metabolites.

Total initial nitrogen amount

Our present investigation showed that *P. sepium* adventitious root growth and periplocin accumulation were restrained at higher initial total nitrogen amount. This result was consistent with the results of Wang and Tan (2002) who also found that the growth and accumulation of artemisinine in the hairy roots were inhibited under higher initial total nitrogen amount. As a general rule, low osmotic pressure is usually favorable to root growth (Baque, Lee, and Paek, 2010; Wu *et al*, 2006; Yu, Hahn, and Paek, 2000). Nitrogen source, as the main inorganic salt, provides salt strength which can maintain a certain amount of osmotic pressure. Therefore, low osmotic environment can be formed by reducing total nitrogen amount. Besides, earlier studies had shown that reduction of nitrogen nutrient in medium usually stimulated the accumulation of secondary metabolite (Phillips and Henshaw, 1977; Knobloch and Berlin, 1983). In the study of *C. acuminata*, Li (2002) reported that camptothecin concentration of *C. acuminata* hydroponic seedlings increased after nitrogen deficiency and he hypothesized that the nitrogen deficiency increased camptothecin concentration by possibly creating an environmental stress.

Two-stage culture

There are a lot of successful cases on cell suspension cultures by using two-stage culture based on nitrogen source, but few reports on the effect of two-stage culture based on nitrogen source on adventitious root cultures. In our experiment, we obtained satisfactory results to gain high periplocin yield in *P. sepium* adventitious roots by using two-stage culture based on nitrogen source. Furthermore, though with poor proliferation ability, *P. sepium* adventitious roots grew thicker after they were directly transferred from the growth medium to production medium, which increased the root biomass greatly. This phenomenon was different from that in cell suspension cultures. Cell suspension cultures usually could not obtain more biomass after they were removed to the production medium. This was mainly because the biomass accumulation of suspension cells was often obtained only by cell proliferation. So after suspension cells were transferred into production medium, the inhibited proliferation ability often led to unconspicuous improvement in biomass.

Besides, in the experiment, we also found that the adventitious roots were cut into segments and then transferred into production medium, the root segments could not grow thick obviously but only generated a small quantity of lateral roots instead (Fig. 2C). The reason might be that the physiological status of adventitious roots was changed after they were cut into

segments. And this change might lead to different physical reactions to the production medium. This could also explain that why in the experiment of nitrogen source investigation, the dry biomass of adventitious roots (cut into segments) in production medium was much lower than that in growth medium, but the dry biomass of adventitious roots (without being cut into segments) increased significantly after being transferred into production medium directly.

References

Baque MA, Lee EJ, Paek KY, 2010. Media salt strength induced changes in growth, physiology and secondary metabolite content in adventitious roots of *Morinda citrifolia*: The role of anti-oxidant enzymes and phenylalanine ammonia lyase. *Plant Cell Rep* 29: 685-694.

Bensaddek L, Gillet F, Saucedo JEN, Fliniaux M, 2001. The effect of nitrate and ammonium concentrations on growth and alkaloid accumulation of *Atropa belladonna* hairy roots. *J Biotechnol* 85: 35-40.

Chen YC, Yi F, Cai M, Luo JX, 2003. Effects of amino acids, nitrate, and ammonium on the growth and taxol production in cell cultures of *Taxus yunnanensis*. *Plant Growth Regul* 41: 265-268.

Chen YP, Li GX, Wu P, Han KJ, Sun XY, Cui SZ, 2005. The reason and precaution of clinically adverse reaction of *Coxtex Periplocae Radicis*. *Chin Hosp Pharm J* 25(7): 689-690.

Crawford NM, 1995. Nitrate: Nutrient and signal for plant growth. *Plant Cell* 7: 859-868.

Cui XH, Murthy HN, Wu CH, Paek KY, 2010. Adventitious root suspension cultures of *Hypericum perforatum*: Effect of nitrogen source on production of biomass and secondary metabolites. *In Vitro Cell Dev Biol Plant* 46: 437-444.

Huang T, Gao WY, Wang J, Cao Y, Zhao YX, Huang LQ, Liu CX, 2010. Selection and optimization of a high-producing tissue culture of *Panax ginseng* C. A. Meyer. *Acta Physiol Plant* 32: 765-772.

Kit SM, 1964. Comparative biological effects of strophanthin-k, corelborin-p, cymarin and periplocin on the cat heart in lesions induced with zinc hydroxide. *Farmakol Toksikol* 27: 699-701.

Knobloch KH, Berlin J, 1983. Influence of phosphate on the formation of the indole alkaloid and phenolic compounds in cell suspension cultures of *Catharanthus roseus* I. Comparison of enzyme activtities and product accumulation. *Plant Cell Tiss Org Cult* 2: 333-340.

Li ZH, 2002. Effects of several abiotic and biotic factors and plant hormones on growth, morphology, and camptothecin accumulation in *Camptotheca acuminata* seedlings. Louisiana State University, Baton Rouge, USA.

Liu H, Pan GX, Gao XM, Zhang BL, 2004. Determination of periplocin in *Periplocae Cortex* by HPLC. *Chin Tradit Herb Drugs* 35(5): 578.

Moreno PRH, van der Heijden R, Verpoorte R, 1995. Cell and tissue cultures of *Catharanthus roseus*: A literature survey. II. Updating from 1988 to 1993. *Plant Cell Tiss Org Cult* 42: 1-25.

Pan XW, Xu HH, Liu X, Gao X, Lu YT, 2004. Improvement of growth and camptothecin yield by altering nitrogen source supply in cell suspension cultures of *Camptotheca acuminate. Biotech Lett* 26: 1745-1748.

Panda AK, Mishra S, Bisaria VS, 1992. Alkaloid production by plant cell suspension cultures of *Holarrhena antidysenterica*: I. Effect of major nutrients. *Biotechnol Bioeng* 39: 1043-1051.

Phillips R, Henshaw GG, 1977. The regulation of synthesis of phenolics in stationary phase cell cultures of *Acer pseudoplatanus* L. *J Exp Bot* 28(4): 785-794.

Ren XL, Liu H, Qi AD, Wang Y, 2007. Improving of simultaneous determination of periplocin and 4-methoxy salicylic aldehyde in *Coxtex Periplocae* with HPLC method. *Tianjin J Tradit Chin Med* 24(3): 252-254.

Umehara K, Sumii N, Satoh H, Miyase T, Kuroyanagi M, Ueno A, 1995. Studies on differentiation inducers. V. Steroid glycosides from *Periplocae Radicis Cortex*. *Chem Pharm Bull* 43: 1565-1568.

Wang JW, Tan RX, 2002. Artemisinin production in *Artemisia annua* hairy root cultures with improved growth by altering the nitrogen source in the medium. *Biotech Lett* 24: 1153-1156.

Wang Q, Ren XL, Wang Y, Pan GX, Gao XM, Qi AD, 2008. Study on excretion of periplocin in rats. *J Tianjin Univ Tradit Chin Med* 27(1): 29-32.

Wang XY, Gao XM, Liu H, Liu Y, Jiang M, Hu LM, Zhang BL, 2010. Gene expression profiling of the proliferative effect of periplocin on mouse cardiac microvascular endothelial cells. *Chin J Integr Med* 16(1): 33-40.

Wu CH, Dewir YH, Hahn EJ, Paek KY, 2006. Optimization of culturing conditions for the production of biomass and phenolics from adventitious roots of *Echinacea angustifolia*. *J Plant Biol* 49: 193-199.

Xu LQ, Lu HZ, Zhang YL, 1998. Clinical observation on treatment of 147 cases of chronic congestive heart failure by *Bei Wujiapi* mixture. *Yunnan J Tradit Chin Med Mat Med* 19(4): 29-31.

Yu KW, Hahn EJ, Paek KY, 2000. Production of adventitious ginseng roots using bioreactors. *Korean J Plant Tiss Cult* 27: 309-315.

Zhang YH, Zhong JJ, Yu JT, 1996. Effect of nitrogen source on cell growth and production of ginseng saponin and polysaccharide in suspension cultures of *Panax notoginseng*. *Biotechnol Prog* 12: 567-571.

Zhang J, Yang G, Shan BE, Zhang C, Zhao RN, Liu JH, 2010. Antitumor effect of periplocin from *Periplocae Cortex* on H_{22} tumor-bearing mice and its mechanism. *Chin Tradit Herb Drugs* 41(8): 1307-1311.

Zhou FJ, Xie YS, Xiao XF, Xu YY, Zhang ZF, Liu CX, 2011. Pharmacokinetic studies of periplocin in rats after intracenous injection. *Drug Eval Res* 34: 81-84