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DIRECT PLANT REGENERATION FROM STEM EXPLANTS OF *SWAINSONA SALSULA* TAUBERT: THE STIMULATORY EFFECT OF SILVER NITRATE AND SUCROSE ON SHOOT INDUCTION

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Abstract

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An efficient regeneration system for large-scale propagation of *Swainsona salsula* Taubert was developed using stem explants. Stem segments were cultured on Murashige and Skoog's medium containing BA, NAA, and silver nitrate in various concentrations, and it was found adventitious shoots regenerated directly from explants on most media with silver nitrate. The best response in terms of frequency of shoot induction (79.62%) and shoot number per explant (6.32) was observed on medium supplemented with 3.0 mg·L^{-1} BA, 0 mg·L^{-1} NAA, and 2.0 mg·L^{-1} $AgNO_3$. Based on the optimal combination of the aforementioned three factors, single factor experiment showed that 60g·L^{-1} sucrose was optimum for shoot induction. The rate of shoots induction rose up to 86.67%, and the average shoot number increased from 6.32 to 8.36. After transferred to half strength MS medium, 85.14% shoots rooted easily on medium with 2.0mg·L^{-1} IBA. In vitro propagated plants could be transferred to soil with 82.5% survival rate.

Key words: Swainsona salsula Taubert · stem explant · silver nitrate · regeneration · *in vitro* ·sucrose
Abbreviations: BA: 6-benzylaminopurine; NAA: α-naphthalene acetic acid; Suc: sucrose; IBA: Indole-3-butyric acid; GA_3: Gibberellin A_3; MS: Murashige and Skoog (1962) medium; $AgNO_3$: silver nitrate

Introduction

Swainsona salsula Taubert, a member in Papilionoideae family, widely distributes in the desert regions of northwestern China, Mongolia and Siberia. As a Chinese medicinal herb, it is included in Compilation of the National Chinese Herbal Medicine (Writing Group of National Chinese Herbal Medicine, 1996). The whole plant could be used for diuresis, hemostatic and treating nephritis, chronic hepatitis and cirrhosis ascites edema. Research results proved the extract of this plant contains many alkaloids and one of the major alkaloids, swainsonine, could inhibit the synthesis of N-linked oligosaccharide in the malignant tumor cells, increasing tumor cell sensitivity to natural immunity (Dennis and Laferte, 1987; Goss, 1997). *S. salsula* is also used for windbreak and sand-fixation to prevent desertification in many areas. Because of high protein content, this plant has a

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high nutritional value, while it is poisonous as fodder for animals, greatly limiting its economic value (Zhang, 1999; Niu and Jiang, 2004).

To increase the content of swainsonine and the degree of tolerance to salt stress and eliminate its toxic, agrobacterium-mediated transformation will be a good method to improve its traits. However, the limiting step to the successful use of the techniques in genetic improvement is not transgene insertion itself, but rather the regeneration of viable plants from the transgenic explant material (Murphy, 2003). So a high-frequency plantlet regeneration system was necessary to be established as the base.

Using cotyledons and hypocotyls as explants, the regeneration system had been successfully established (Yang et al., 2001). In this experiment, the stem segment, the relatively elder part of the plant, was used as the explants and a more efficient plant regeneration system was set up via mainly testing the effects of phytohormones, silver nitrate and sucrose on shoot regeneration. Meanwhile, different from previous report in *S. salsula* (Yang et al., 2001), the positive effects of proper concentration of silver nitrate and sucrose on shoot induction were found.

Materials and Methods

Explant preparation

The seeds of *S. salsula* collected from Minqin County, Gansu Province were immersed in 98% H_2SO_4 for 20 min. Then they were flushed under tap water for at least 30 min to wash away the H_2SO_4 attached to the surface. The sterilization was performed by immersing seeds in 0.1% mercuric chloride solution for 10 min providing occasional shaking. Then the mercuric chloride solution was drained out and the materials were thoroughly washed four to five times with sterile distilled water. After being surface sterilized, disinfected seeds were inoculated on MS (Murashige and Skoog, 1962) basal medium supplemented with 3% sucrose and without growth regulators for germination. One month later, the stem of the aseptic seedlings were used for experiment.

Shoots induction

Stem from the aseptic seedlings were excised 5-6 mm segments and then placed on medium for shoot induction. The effect of four factors including BA, NAA, $AgNO_3$ and sucrose were studied by two experiments successively, a full factorial experiment and a single factor test, and each of the factors was set at three levels (Table 1). In the full factorial experiment, 27 treatments were carried out to study the effect of BA, NAA and $AgNO_3$ for obtaining the optimal combination of their levels (Table 2). And then the optimal level of the sucrose was decided based on the optimal combination of BA, NAA and $AgNO_3$ in the single factor test (Table 3). All the experiments were repeated three times. For each treatment, five explants were cultured per flask and the numbers of shoots of 40 randomly chosen explants per repetition were tested. MS medium free of hormone was used as a control. After incubation for 40 days in each experiment, shoot regeneration frequency and mean shoot number based on total explants were calculated and recorded, respectively.

Shoots elongation

After 40 days, the shoot clusters were cut into small parts and placed on MS medium with or without phytohormone for elongating. Different growth regulators (BA, GA_3) in various combinations were used in MS medium to test their effects on shoot elongation. Two weeks later, the states

Table 1

Factors and level for shoot induction

Level	Factor			
	BA, $mg \cdot l^{-1}$	NAA, $mg \cdot l^{-1}$	$AgNO_3$, $mg \cdot l^{-1}$	SUC, $g \cdot l^{-1}$
1	2	0	0	15
2	2.5	0.1	2	30
3	3	0.5	4	60

Table 2

The effect of different combination of BA, NAA and $AgNO_3$ on shoot induction

Treatment	Factors, $mg \cdot l^{-1}$			Shoots induction rate, %	Shoots NO. per explant
	BA	NAA	$AgNO_3$		
1	1	1	1	38.33 ± 0.83def	1.19 ± 0.13e
2	2	2	2	33.33 ± 1.92ef	0.97 ± 0.12ef
3	3	3	3	27.78 ± 6.19fgh	0.68 ± 0.21fg
4	1	1	3	18.89 ± 4.00hijk	0.38 ± 0.16
5	2	2	1	0.83 ± 0.83no	0.03 ± 0.02h
6	3	3	2	16.67 ± 3.33ijkl	0.33 ± 0.13gh
7	1	1	2	38.89 ± 1.11de	1.34 ± 0.36e
8	2	2	3	7.28 ± 1.43lmno	0.10 ± 0.05h
9	3	3	1	0.00 ± 0.00o	0.00 ± 0.00h
10	1	3	2	8.89 ± 2.22jklmno	0.09 ± 0.02h
11	2	1	3	30.83 ± 3.00efg	1.20 ± 0.18e
12	3	2	1	13.33 ± 1.92ijklm	0.14 ± 0.09h
13	1	3	1	0.00 ± 0.00o	0.00 ± 0.00h
14	2	1	2	56.67 ± 5.09b	3.87 ± 0.15b
15	3	2	3	5.00 ± 1.44mno	0.49 ± 0.09gh
16	1	3	3	55.93 ± 4.43b	2.25 ± 0.09c
17	2	1	1	20.00 ± 3.85hij	0.41 ± 0.22gh
18	3	2	2	21.11 ± 2.94ghi	0.42 ± 0.11gh
19	1	2	3	14.44 ± 1.11ijklm	0.30 ± 0.07gh
20	2	3	1	0.00 ± 0.00o	0.00 ± 0.00h
21	3	1	2	79.62 ± 2.10a	6.32 ± 0.17a
22	1	2	2	52.50 ± 7.50bc	1.86 ± 0.09cd
23	2	3	3	44.44 ± 5.88cd	2.30 ± 0.25c
24	3	1	1	5.56 ± 2.22lmno	0.06 ± 0.02h
25	1	2	1	7.78 ± 2.94klmno	0.19 ± 0.11gh
26	2	3	2	11.67 ± 4.41ijklmn	0.31 ± 0.02gh
27	3	1	3	31.11 ± 5.56efg	1.43 ± 0.31de

[a]Values are mean ± standard error of three repeated experiments with about 40 explants used in each treatment.

[b]Means followed by the same letters are not significantly different according to the one-way ANOVA Duncan's test at the 5% level of significance.

of elongated shoots in different treatments were described. All treatments were repeated ten times and the experiment was repeated twice.

Rooting of shoots and acclimatization

The regenerated healthy shoots about 1-2 cm were transferred to the half strength MS medium

Table 3
The effect of Sucrose concentration on shoot induction

Sucrose, $g \cdot l^{-1}$	Shoots induction rate, %	Shoots NO. per explant
15	70.92 ± 3.17	4.94 ± 0.13
30	78.94 ± 2.10	6.26 ± 0.17
60	86.67 ± 1.67	8.39 ± 0.32

[a]Values are mean ± standard error of three repeated experiments with about 40 explants used in each treatment

supplied with 0.5, 1.0, 1.5, or 2.0 $mg \cdot l^{-1}$ IBA for rooting. MS medium free of IBA was used as control. In each treatment, thirty single shoots were used and all treatments were repeated three times. One month later, the root induction rate, the mean numbers and length of roots were recorded. Following root initiation, the regenerated whole plantlets with developed roots were washed with sterile water to remove agar from their roots, then transferred to pots containing autoclaved mixture of the agricultural substrate plantamax and sand (1:1), covered with polythene bags to maintain high humidity and kept at 25 ± 2°C in a growth chamber. After 1 week, the bags were perforated, and the plants were transferred to the green house.

Culture medium and conditions

The pH of all media was adjusted to 5.7 with 1 $mol \cdot l^{-1}$ NaOH, then all the media were solidified with 0.7% agar prior to autoclaving at 105 kPa, 121°C for 15 min. The growth regulators were supplied before autoclaving, while the $AgNO_3$ was filter sterilized and added to the medium after autoclaving. All cultures were incubated in 150 ml vitreous culture flasks each containing 25 ml of medium and placed at 25±2°C in a culture room with 16 h photoperiod under cool-white fluorescent light of approximately 3000 lx.

Data collection and analysis

After the data were collected from all the treatments for shoot induction, they were used to calculate the average of shoot induction rate and mean shoot number based on total explants, and then the means were compared using Duncan's multiple range tests (95% confidence level). In all experiments, the standard errors of means were calculated.

Results

Shoots induction

After 10 days of culture, the cut ends of the stem-segment explants began to expand (Figure A). Many wart like structure or shoot primordia could be observed under Stereo Microscope at one or both cut ends of the stem sections on 15 days of culture (Figure B). 25 days after inoculation, visible intense adventurous shoots appeared, and they grew around the cut surface of the stems on the shoot induction medium (Figure C). Following another 15 days, it was found adventitious shoots regenerated directly from explants on most media with silver nitrate, and most shoots were normal and healthy, but few abnormal shoots appeared with only enlarged leave and could not elongate (Figure D).

No shoots regenerated when stem explants were cultured on MS basal medium alone. Vitrification of shoots was observed in the medium containing BA alone, but it could be controlled by adding proper amount (2.0 $mg \cdot l^{-1}$) of $AgNO_3$ in the medium effectively. As list in Table 2, Duncan's multiple range tests showed when stem explants cultured on MS medium containing 3.0 $mg \cdot l^{-1}$BA,

0.0 mg·l^{-1}NAA and 2.0 mg·l^{-1}$AgNO_3$, the optimal shoot induction rate (79.62%) and shoot number per explant (6.32) was obtained, while explants on medium with only 3.0 mg·l^{-1}BA, 0.0 mg·l^{-1}NAA could achieve 5.56% induction rate and 0.06 shoot number respectively, showing $AgNO_3$ could dramatically promote shoot induction. However, the excess $AgNO_3$ (4.0 mg·l^{-1}) resulted in the decline in the rate of shoots induction or no regeneration shoots.

To further improve the induction rate and the number of the shoots, a sample single factor test was performed to gain the optimum level of the sucrose based on the above optimal medium. The results showed 60g·L^{-1} sucrose was more efficient than 15g·L^{-1} sucrose and 30g·L^{-1} sucrose. The

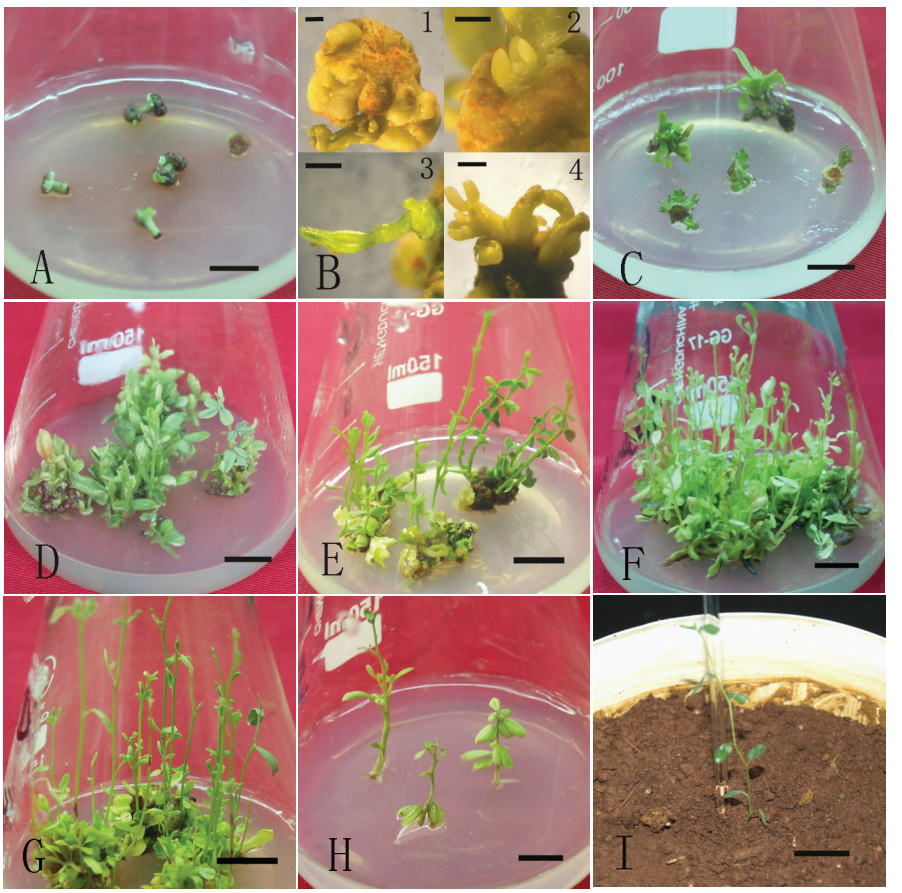

Fig. 1. Stages of direct shoot bud induction, elongation and regeneration in S. salsula Taubert: A- Explants showing morphogenic responses on the MS medium containing 3.0mg•l^{-1}BA, 2.0mg•l^{-1} $AgNO_3$ and 60.0g•l^{-1} sucrose (bar =1cm); B- The process of adventitious buds formation at the microscopic level: 1- wart like structure 2- Normal shoot primordial. 3- Abnormal shoot primordia with enlarged leaf primordia. 4- Many different shoot primordias (bar = 1 mm); C- Formation of adventitious buds on the view of macroscopic (bar = 1 cm); D- Shoot development from induced buds (bar = 1 cm); E- Shoot elongation on the base MS medium without any phytohormone (bar = 1 cm); F- Shoot elongation on the MS medium adding 0.3 mg•l^{-1} GA_3 (bar = 1 cm); G- Shoot elongation on the MS medium including 0.1 mg•l-1 GA_3 and 1.5 mg•l^{-1} BA (bar = 1 cm); H- Root formation on the 1/2 MS medium supplement with 2.0mg•l^{-1} IBA (bar = 1 cm); I- Plants established after transplantation (2 weeks) to plastic flower pot (bar = 1 cm).

rate of shoots induction rose up to 86.67%, and the average shoot number increased from 6.32 to 8.36 (Table 3). So, the final optimal combination for shoots regeneration was determined as MS medium with 3.0 mg·l^{-1}BA, 0.0 mg·l^{-1}NAA, 2.0 mg·l^{-1}$AgNO_3$, and 60 g·l^{-1} sucrose.

Shoots elongation

As the shoot bushes obtained direct from shoot induction medium were too dense which influenced the shoot elongation seriously, they were cut into small parts and transferred on MS medium with or without plant hormones for elongation. 15 days later, normal regeneration shoots in MS base medium elongated to 1.0-3.0 cm in length and based on an overall consideration of the shoots state of various treatments, the base MS with free grow regulator was the most ideal medium for shoot elongation.

Rooting of shoots and acclimatization

When shoots grew up to 2.0-3.0 cm in length, they were separated individually from the explants and grown on MS medium with or without IBA. No roots developed from shoots on MS medium without IBA. The presence of IBA could promote root formation and regenerated shoots rooted with a frequency of 79.76% and 3.52 roots per shoot on 2.0 mg·l^{-1} IBA supplemented medium (Figure H)(Table 4). After 4 weeks, plantlets developed

Table 4

Effect of IBA concentration on root formation

IBA	Root induction rate, %	Root length	Root NO.
0	0.00±0.00	0.00±0.00	0.00±0.00
0.5	25.24±4.82	23.96± 1.89	1.47± 0.29
1	33.33±2.17	18.66± 2.64	2.08± 0.27
1.5	56.48±2.35	13.66± 3.34	2.16± 0.16
2	79.76±1.37	10.68± 1.46	3.52± 0.11

[a]Values are mean ± standard error of three repeated experiments with about 30 explants used in each treatment. Experiments with about 30 explants used in each treatment

could grow healthily, while the abnormal shoots couldn't elongate and got to death finally (Figure E). On medium containing GA_3, the normal and abnormal shoots could elongate, but the elongated shoots were unhealthy thin, weak, few leaves, withered tip buds (Figure F). Meanwhile with increasing the concentration of GA_3 from 0.1 mg·l^{-1} to 0.3 mg·l^{-1}, the unhealthy of the shoots became more serious. The addition of BA could release the effect of GA_3 on shoots and could shorten the height of the shoots and thicken the leaves of shoots with increasing the BA concentration, but couldn't inhibit the wilt of the tip buds (Figure G). Therefore the medium containing BA and GA_3 was not suitable for elongating the shoots. Finally, a strong root system. Finally, through gradual acclimatization, 82.5% regenerated plants were successfully grown and no obvious phenotypic variations were observed (Figure I).

Discussion

Yang et al. (2001) tried to regenerate *S. salsula* by using different cytokinin, BA and TDZ, or various combinations of them with NAA, and eventually gained an optimum medium for shoot induction successfully by using the cotyledon explants. But a primary test showed the same condition as for cotyledon could not induce adventitious buds for the stem explants, relatively

elder explants. This might be the different physiological state of different explants, resulting in different response to culture conditions (Kamada et al., 1995).

BA, alone or in combination with NAA, is known to be an effective shoot inducing agent in different species (Blakesley and Constantine, 1992). In our study, BA alone could induce few shoots with serious vitrification and the combination of BA and NAA promoted the callus formation. Therefore it was clear that the medium only supplemented with BA alone or BA and NAA was not suitable to induce the regeneration shoots.

The widespread effectiveness of ethylene inhibitors at promoting shoot organogenesis in several plant species has been reviewed by Kumar et al. (1998), and silver nitrate is the most commonly used source of silver ion which could interfere with ethylene incorporation at its receptor sites (Beyer, 1979; Chi et al., 1990). In this experiment, when $AgNO_3$ was added into BA supplemented medium, it was found that the vitrified shoots formation was apparently inhibited, and the right amount of $AgNO_3$ into the medium containing single BA or with NAA dramatically improved the shoot induction rate and shoot number. According to prevail reports, vitrification of shoots is related to an elevated ethylene production which was related to hypolignification and poor cell wall, and addition of $AgNO_3$ as a vitrified shoots inhibitor may be due to the interference with ethylene (Ziv, 1991). Furthermore, adding $AgNO_3$ to medium containing BA and NAA could weaken the formation of callus in explants, similar to prevail report in shoot regeneration from leaflets of peanut (Zhou and Zhang, 2002), which might be the inhibition of release of ethylene that enhancing callus formation (Robinson et al., 1987).

Sucrose is the most commonly used carbon source in heterotrophic and/or mixotrotrophic tissue culture. The effect of sucrose on shoot induction has been reported for several plants (Ambrosio and de Melo, 2004; Karim et al., 2003). In the single factor test, the sucrose concentration dramatically affected the shoot formation of *S. salsula*, and an increase in the shoot regeneration rate and shoot number was observed when concentration of sucrose increased to 60 g/l^{-1}. The sucrose is not only an osmotic regulator, but also a nutrient and a growth regulator (Samoylov et al., 1998). Sucrose increased enzyme activities as has been reported for phosphoenolpyruvate carboxylase activity of in vitro *Solanum tuberosum* plantlets (Boubacar et al., 2001).

Shoot elongation and development have been improved by the use of GA_3 (Dong and Jia, 1991; Power, 1987; Schrammeijer et al., 1990). In our experiment, low concentration GA_3 resulted in over-elongation of shoots, similar to previous report in *Helianthus annuus* (Paterson, 1984), but the phenomenon could be controlled by adding BA into the medium containing GA_3. However, whether or not used together with BA, the tip of the shoots died and it may be related to the preferential action of GA in young, meristematic cells (Kende and Zeevaart, 1997). Thus the result was the MS base medium without any phytohormone was enough to elongate the shoots and to eliminate the effects of growth regulators in the previous culture in this experiment, similar to previous report. (Qin et al., 2006).

Conclusion

In conclusion, an efficient regeneration protocol was established from stem explants of *S. salsula*. The medium supplemented with 3.0 $mg \cdot l^{-1}$ BA, 0 $mg \cdot l^{-1}$ NAA, 2.0 $mg \cdot l^{-1}$ $AgNO_3$ and 60 $g \cdot l^{-1}$ sucrose was used to induced shoots. The inducted shoots were transferred on MS base medium for further elongation. Half strength MS containing 2.0 $mg \cdot l^{-1}$ IBA was most efficient in rooting. The plantlet with develop roots was transferred successfully to greenhouse by gradual acclimatization.

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