Adventitious Bud Induction and Plant Regeneration from Stem Nodes of *Salvia splendens* ‘Cailinghong’

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Abstract. *Salvia splendens* is a widely used ornamental bedding plant; however, the limited propagation method has decreased its quality and yield. Through years of selection, we have obtained a new variety of *S. splendens* with weak apical dominance and named it as ‘Cailinghong’. To establish an effective method for regeneration of *S. splendens* ‘Cailinghong’, different explants, including leaves, receptacles, petioles, stem nodes, and stem segments were used for adventitious bud induction. Next, various combinations of plant growth regulators (PGRs) were selected for bud and root induction, which were assessed by adventitious bud initiation rate and proliferation rate, as well as root induction rate. Meanwhile, the survival rate of transplanted plantlets was also calculated. As a result, stem nodes were found easy to be induced to form buds, and the optimum medium component was 1/2 Murashige and Skoog (MS) medium supplemented with 0.45 μM naphthalene acetic acid (NAA), 8.88 μM 6-benzylaminopurine (6-BA), and 2.46 μM 3-indolebutyric acid (IBA) for plantlets induction, whereas 1/4 MS medium supplemented with 2.23 μM NAA for root induction. Furthermore, the survival rate of transplanted plantlets was up to 80%, and all regenerated plantlets were normal in phenotype. Therefore, cultured in 1/2 MS medium with combined PGRs, whole plantlet of *S. splendens* ‘Cailinghong’ could be regenerated directly from stem node.

Among all the numerous ornamental bedding plants, due to its bright colors and long florescence, *S. splendens* belonging to Labiatae family, is most widely planted in gardens, flowerbeds, and by roadsides (Soundararajan et al., 2013). With the development of research and technology, the breeding of *S. splendens* has been largely improved (Liu et al., 2012), and many new varieties have been cultivated, such as *S. splendens* ‘Zhongchuanhong’, a tall variety breed (Hui et al., 2004), and *S. splendens* ‘Shenzhouhong’, a variety produced through space breeding (Fu et al., 2009).

It has been reported that, wild type *S. splendens* as well as its new varieties need to remove the terminal bud three or four times to

meet the product requirements (Zhi et al., 2013). Through years of selection, we have found a new variety with weak apical dominance and named it as ‘Cailinghong’. Its original lateral branch develops into the first lateral branch during vegetative growth phase, which further develops into multilevel lateral branches. ‘Cailinghong’ grows and develops without the need of manual removal of the terminal bud during its whole life. At last, it forms a naturally low and spherical type plant with multilevel lateral branches and blossom exuberant, which is more suitable for landscaping. As far as we know, there is no other variety of *S. splendens* having such powerful branching ability as ‘Cailinghong’ does. In addition, related report about its tissue culture remains rare.

However, there are still many problems in the development of *S. splendens*, such as limited colors, serious pests, and diseases, which will inevitably lead to a decrease in yield and a decline in flower quality. What is more, *S. splendens* shows higher requirements on temperature, which adds difficulties on its reproduction (Lai et al., 2001). Thus, it is of great importance to investigate proper rapid propagation technique of *S. splendens*.

Recently, an indirect regeneration method that uses the callus of *S. splendens* has been reported. Unfortunately, the regeneration cycle is comparatively long. Moreover, under in vitro conditions, because of long-term exposure to PGRs and chemicals, the callus cells

are very sensitive (Maruthi Rao et al., 2012), and they can exhibit abnormal morphological characteristics and somaclonal variation. Direct regeneration by organogenesis is an alternative method, referring to the culture of different tissues in vitro such as buds, leaves, stems, and roots, to develop new shoots (Mallón et al., 2011). In all, it is an effective method of mass propagation of plants, while there is no need for callus as an intermediate stage.

In this study, explants (receptacle, leaf, petiole, stem segment, and stem node) were selected to establish an effective method for regeneration of *S. splendens* ‘Cailinghong’. Then the effects of PGRs on adventitious bud induction and shoot regeneration were evaluated. The results may provide clues for further application of biotechnology on regeneration of *S. splendens* ‘Cailinghong’.

Materials and Methods

Plant materials. *S. splendens* ‘Cailinghong’ was purchased from Zhejiang Hongyue Flower Co. Ltd (Hangzhou, Zhejiang, China). Explants (receptacles, leaves, petioles, stem nodes, and stem segments) were obtained from 2- to 4-month-old mature ‘Cailinghong’ grown in the greenhouse (22 $\pm$ 2 °C with humidity of 60%).

Selection of explants. Receptacles were obtained from flower bud whose height was about 1 cm. Leaves, petioles, and stems were collected from tender branches and leaves of the collected stem nodes were removed. Then explants of leaf were cut into small pieces of 5 mm $\times$ 5 mm, and explants of petiole, stem node, and stem segment were cut into small pieces of 5 to 9 mm. In this way, each stem node only contained two axillary buds. Explants were surface sterilized by 75% (v/v) ethanol for 30 s, then by 1% (v/v) sodium hypochlorite for 1 min, and finally rinsed with sterile distilled water for three times (3 min each time). Three explants of receptacle, leaf, petiole, stem node, or stem segment were planted in each glass vessel (five glasses for each type of explant). All explants were cultured in glass vessels that contained 1/2 MS basal culture medium (Murashige and Skoog, 1962) without PGRs. The initiation rate was observed and calculated: Initiation rate = (No. of initiated explants/No. of inoculated explants) $\times$ 100%.

Induction of adventitious bud. By calculating the initiation rates of different types of explants, the explant with the highest initiation rate was chosen as the optimal explant. Then the overall experiment of all the possible combinations was designed and the sterilized optimal explants were placed on 1/2 MS medium (pH 5.8) supplemented with different concentrations and combinations of NAA (0, 0.45, or 2.23 μM), 6-BA (4.44, 8.88, or 22.20 μM), and IBA (0, 2.46, or 4.92 μM) to determine the optimum medium for adventitious bud induction (Soundararajan et al., 2013). The concentrations of 13.32 and 17.76 μM 6-BA were excluded because

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there were no significant changes at 13.32 and 17.76 μM when compared with 8.88 μM 6-BA in the pre-experiment. In total, 27 treatments were generated and 3 replicates (15 explants for each replicate) were performed for each treatment. After 3 weeks of tissue culture, the differentiation rate and proliferation rate were calculated to measure the growth conditions of explants and confirm the optimal concentration and combination of NAA, 6-BA, and IBA. Differentiation rate = (No. of differentiated explants/No. of inoculated explants) $\times$ 100%. Proliferation rate = (No. of buddings/No. of inoculated explants) $\times$ 100%. All the chemical reagents were purchased from Sigma-Aldrich GmbH (Munich, Germany), and the medium was first sterilized using the MLS-3780 autoclave (Sanyo, Japan) before being used. The culture condition was 14 h photoperiod with illumination of 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at $25 \pm 2^\circ\text{C}$, 60% RH (relative humidity).

Plant regeneration. Adventitious buds were cultured in the same medium for subculture after induction from explants for 3 to 5 weeks. When the regenerated shoots grew to 2 to 3 cm (about three leaf segments), they were transferred to the autoclaved inducing medium and cultured under 16 h photoperiod with illumination of 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at $25 \pm 2^\circ\text{C}$, 60% RH. The medium contained 1/4 MS medium that was supplemented with various concentrations of NAA (0, 2.23, 4.46, 11.15, or 22.29 μM). Root induction frequency was recorded and calculated: Root induction frequency = (No. of rooted explants/No. of inoculated explants) $\times$ 100%.

After 5 to 6 weeks, when the height of plantlets reached above 8 cm and root length over 3 cm, they were moved into plastic pots (70 mm $\times$ 65 mm $\times$ 80 mm) in greenhouse

(22 to 26 $^\circ\text{C}$) with culture medium for 2 d. After removing agar from roots by gentle wash, the plants were transplanted into pots containing vermiculite and perlite (v/v = 2/1), which had been autoclaved at 121 $^\circ\text{C}$ under 1.1 $\text{kg}\cdot\text{cm}^{-2}$ pressure for 60 min. Two weeks later, the survival rate of transplants was calculated and the survived plants were transplanted into larger garden pots (130 mm $\times$ 90 mm $\times$ 110 mm). Survival rate = (No. of survived plants/No. of plants moved into pots in greenhouse) $\times$ 100%.

Statistical analysis. All the experiments were repeated three times and the data were expressed as mean $\pm$ SE. Differences between the means were compared by analysis of variance (ANOVA) followed by Duncan's multiple range tests (Duncan, 1955) at the 0.05 level, using SAS V8.02 (SAS Institute Inc., Cary, NC). Quadratic regression analysis was also performed to detect the induction effect changing with PGRs concentrations.

Results and Discussion

Selection of explants. Our results showed that leaves exhibited no significant morphological changes, and formed neither buds nor callus; whereas receptacles, petioles, stem nodes, and stem segments exhibited significant morphological changes, formed small buds and enlarged stem bases (Table 1). In addition, stem segments could slowly form a few buds and could easily brown. Furthermore, the initiation rate of stem nodes was the highest (93.45%) (Fig. 1A), that of stem segments was the second highest (65.25%), and that of petioles was the lowest (15.95%) among all explants. Previous study has shown that the stem nodes with axillary buds have several advantages,

such as easy operation, maintenance of the stability of important agronomic traits, short regeneration cycle, and high regeneration rate, and have been widely used in tissue culture (Han et al., 2006; Zhang et al., 2010). Therefore, stem node was the most suitable explant for adventitious bud induction.

Effects of PGRs on bud induction. Different combinations of PGRs were adopted to investigate the optimum culture medium for bud formation. The results showed that the differentiation and proliferation rates of stem nodes increased significantly with the increase of 6-BA concentration from 4.44 to 8.88 μM . However, when the concentration further increased to 22.20 μM , the differentiation and proliferation rates decreased significantly (Table 2). The quadratic regression equations were: differentiation rate = $20.283 \times 6\text{-BA} + 11.129 \times \text{IBA} - 0.737(6\text{-BA})^2 - 60.839$ ($R^2 = 0.857$); proliferation rate = $33.8046 \times 6\text{-BA} + 18.585 \times \text{IBA} - 1.228 \times (6\text{-BA})^2 - 101.492$ ($R^2 = 0.857$). In addition, the variance analysis indicated that stem nodes showed the highest differentiation rate in mediums supplemented with 0.45 μM NAA, 8.88 μM 6-BA, and 2.46 μM IBA

Table 1. The selection of explants.

Explants	Initiation rate (%)	Characteristics
Receptacle	23.2 $\pm$ 1.4 d	Few small buds
Leaf	31.7 $\pm$ 1.2 c	No obvious change
Petiole	16.0 $\pm$ 1.1 e	Few small buds
Stem node	93.5 $\pm$ 1.1 a	Some small buds
Stem segment	65.3 $\pm$ 1.3 b	Stem base enlarged

Values presented as mean $\pm$ SE, differences were compared with Duncan's multiple range test, and the values followed by the same letter are not significantly different ($\alpha = 0.05$).

Fig. 1. Plant regeneration from stem nodes of 'Caillinghong'. (A) Bud induction from stem nodes with axillary buds. (B) Induced buds of 'Caillinghong'. (C) Shoot proliferation of 'Caillinghong'. (A), (B), and (C) were all cultured in 1/2 MS medium supplemented with 0.45 μM naphthalene acetic acid (NAA), 8.88 μM 6-benzylaminopurine, and 2.46 μM 3-indolebutyric acid. (D) Root induction from the regenerated plantlets. (E) Whole plants regenerated in the root-inducing medium. (D) and (E) were cultured in 1/4 MS medium supplemented with 2.23 μM NAA. (F), (G), and (H) regenerated plants grow in a greenhouse.

(Table 3; Fig. 1B and C). Combining with quadratic analysis and variance analysis, our results indicated that 6-BA ($P < 0.01$) was the most important PGR and IBA ($P < 0.05$) was the second most, whereas NAA ($P = 0.676$) showed no significant effects on differentiation and proliferation rates of *S. splendens*. Thus, we could infer that 6-BA and IBA play important roles in shoot multiplication of *S. splendens*. This is consistent with other studies that showed that 6-BA is effective in bud proliferation inducing in many plant species, such as *Guadua angustifolia* (Jiménez et al., 2006), *Andrographis paniculata* (Purkayastha et al., 2008), *Ilex glabra* (Sun et al., 2010), and *Chrysanthemum morifolium* (Song et al., 2011). Cytokinins, including 6-BA, have shown to be able to induce the formation of shoot apical meristems during shoot organogenesis (Sugiyama, 1999). Although 6-BA has been proved to be effective in inducing shoot multiplication in *S. splendens*, it inhibited bud proliferation at high concentration. Similar results have been observed in *Spathiphyllum floribundum* (Werbrouck et al., 1995, 1996). Moreover, because of its stability, IBA was among the list of extensively used auxins as well. It is reported that high ratio of 6-BA and IBA tends to facilitate bud induction, whereas low ratio is favorable for root induction (Guo et al., 2012), which was consistent with our findings. Therefore, stem nodes were easy to be induced to form buds and the medium supplemented with 0.45 μM NAA, 8.88 μM 6-BA, and 2.46 μM IBA was the optimal medium for bud formation and proliferation.

Induction of roots. The regenerated plantlets were transferred into root-inducing medium for root development when they grew to 2 to 3 cm height (Fig. 1D). The results showed that, only 1.3% of regenerated plantlets have initiated roots when cultured in medium without PGRs, whereas 89% to 90% of regenerated plantlets induced roots when cultured in medium supplemented with NAA (Table 4; Fig. 1E). Thus, NAA was effective in root induction, and the supplement of NAA to 1/4 MS medium could significantly raise root induction rate. As there was no significant difference in the rates among different NAA concentrations, 2.23 μM NAA was selected for root induction. The effectiveness of NAA in adventitious root induction has been reported in regenerated shoots of many plant species, including *Adenosma glutinosum* (Linn.) (Tu et al., 2014).

Transplantation of plantlets. There were ≈ 3 to 5 microshoots generated in each pot with a height of 3 to 5 cm. Two weeks later after the transplantation into pots with soil mixed with vermiculite and perlite, the survived seedlings were transplanted into larger garden pots under natural conditions (Fig. 1F). The survival rate of transplanted plantlets was up to 80%, and all regenerated plantlets were normal in phenotype (Fig. 1G and H).

Table 2. Bud differentiation rate and proliferation rate from stem nodes of *Salvia splendens* when cultured in medium with various combinations of plant growth regulators (NAA, 6-BA, IBA).

Treatment	NAA (μM)	6-BA (μM)	IBA (μM)	Differentiation rate (%)	Proliferation rate (%)
1	0	4.44	0	29.0 $\pm$ 0.6 i	48.3 $\pm$ 1.0 i
2	0.45	4.44	0	19.0 $\pm$ 0.6 j	31.5 $\pm$ 1.0 j
3	2.23	4.44	0	19.0 $\pm$ 0.6 j	31.5 $\pm$ 1.0 j
4	0	4.44	2.46	24.0 $\pm$ 0.6 i	40.0 $\pm$ 1.0 i
5	0.45	4.44	2.46	24.0 $\pm$ 0.6 i	40.0 $\pm$ 1.0 i
6	2.23	4.44	2.46	22.7 $\pm$ 0.3 i	37.8 $\pm$ 0.5 i
7	0	4.44	4.92	23.7 $\pm$ 0.3 i	39.4 $\pm$ 0.6 i
8	0.45	4.44	4.92	27.3 $\pm$ 0.3 h	45.6 $\pm$ 0.6 h
9	2.23	4.44	4.92	24.7 $\pm$ 0.3 hi	41.1 $\pm$ 0.6 hi
10	0	8.88	0	47.0 $\pm$ 0.6 e	78.3 $\pm$ 1.0 e
11	0.45	8.88	0	50.7 $\pm$ 0.3 g	84.4 $\pm$ 0.6 g
12	2.23	8.88	0	31.3 $\pm$ 1.5 g	52.2 $\pm$ 2.4 g
13	0	8.88	2.46	82.7 $\pm$ 1.5 b	137.8 $\pm$ 2.4 b
14	0.45	8.88	2.46	85.7 $\pm$ 0.7 a	142.8 $\pm$ 1.1 a
15	2.23	8.88	2.46	81.0 $\pm$ 1.0 b	135.0 $\pm$ 1.7 b
16	0	8.88	4.92	76.7 $\pm$ 0.9 c	127.7 $\pm$ 1.5 c
17	0.45	8.88	4.92	80.0 $\pm$ 0.6 b	133.3 $\pm$ 1.0 b
18	2.23	8.88	4.92	76.7 $\pm$ 1.3 c	127.8 $\pm$ 2.2 c
19	0	22.20	0	23.3 $\pm$ 0.3 gh	38.8 $\pm$ 0.6 gh
20	0.45	22.20	0	30.3 $\pm$ 0.9 gh	50.5 $\pm$ 1.5 gh
21	2.23	22.20	0	31.0 $\pm$ 0.6 f	51.7 $\pm$ 1.0 f
22	0	22.20	2.46	38.3 $\pm$ 0.9 f	63.9 $\pm$ 1.5 f
23	0.45	22.20	2.46	24.0 $\pm$ 2.1 i	40.0 $\pm$ 3.5 i
24	2.23	22.20	2.46	22.3 $\pm$ 1.5 i	37.2 $\pm$ 2.4 i
25	0	22.20	4.92	25.0 $\pm$ 2.1 h	41.7 $\pm$ 3.5 h
26	0.45	22.20	4.92	21.3 $\pm$ 1.5 ij	35.6 $\pm$ 2.4 ij
27	2.23	22.20	4.92	22.0 $\pm$ 1.5 ij	36.7 $\pm$ 2.6 ij

Values presented as mean $\pm$ SE, with different letters within a column to indicate significant differences at the 0.05 level. There are 15 explants for each treatment. Each experiment was repeated three times and the differences between the means were compared by Duncan's multiple range tests.

NAA = naphthalene acetic acid; 6-BA = 6-benzylaminopurine; IBA = 3-indolebutyric acid.

Table 3. Test of between-subjects effects.

Dependent variables	Source	Type III sum of squares	Degrees of freedom	Mean square	F value	Significance
Differentiation rate	Corrected model	12,129.4 ^z	6	2,021.6	17.0	0.000
	Intercept	41,827.1	1	41,827.1	350.7	0.000
	NAA	95.3	2	47.7	0.4	0.676
	6-BA	11,089.0	2	5,544.5	46.5	0.000
	IBA	945.1	2	472.5	4.0	0.036
	Error	2,385.4	20	119.3		
	Total	56,342.0	27			
	Corrected total	14,514.9	26			
	Corrected model	33,682.1 ^y	6	5,613.7	17.0	0.000
	Intercept	116,112.0	1	116,112.07	351.0	0.000
Proliferation rate	NAA	264.4	2	132.2	0.4	0.676
	6-BA	30,780.7	2	15,390.4	46.5	0.000
	IBA	2,637.0	2	1,318.5	4.0	0.035
	Error	6,615.6	20	330.8		
	Total	156,409.7	27			
	Corrected total	40,297.7	26			

^z $R^2 = 0.836$ (adjusted $R^2 = 0.786$).

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NAA = naphthalene acetic acid; 6-BA = 6-benzylaminopurine; IBA = 3-indolebutyric acid.

Conclusion

By inducing stem node, the whole plantlets of *S. splendens* 'Cailinghong' could be rapidly regenerated, without an intervening callus phase. Different PGRs combinations and concentrations exhibited significant effects on bud induction. The optimum condition is 1/2 MS medium supplemented with 0.45 μM NAA, 8.88 μM 6-BA, and 2.46 μM IBA for shoot multiplication, and 1/4 MS medium supplemented with 2.23 μM NAA for root induction.

Table 4. Effect of different concentrations of NAA on adventitious root induction of *Salvia splendens* 'Cailinghong'.

NAA concentrations (μM)	Root induction frequency (%)
0	1.3 $\pm$ 0.2 b
2.23	90.0 $\pm$ 0.2 a
4.46	89.5 $\pm$ 0.3 a
11.15	90.1 $\pm$ 0.6 a
22.29	89.7 $\pm$ 1.9 a

Values presented as mean $\pm$ SE, with different letters within a column to indicate significant differences at the 0.05 level.

NAA = naphthalene acetic acid.

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