

Full Length Research Paper

# Influence of plant growth regulators on axillary shoot multiplication and iron source on growth of *Scrophularia takesimensis* Nakai - a rare endemic medicinal plant

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An efficient protocol for the *in vitro* propagation of *Scrophularia takesimensis*, a rare endemic medicinal plant, is described. Shoot multiplication was induced by culturing nodal explants on MS medium containing 3% (w/v) sucrose, 0.8% (w/v) agar, and different concentrations and combinations of plant growth regulators. The greatest percentage of shoot induction was achieved when nodal explants were cultured on MS medium supplemented with 2.0 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> IAA with an average of 16 shoots per explant. The microshoots were separated from the multiple shoots and sub-cultured onto MS medium with 3% (w/v) sucrose and 0.8% (w/v) agar for further growth, and rooting. The plantlets growth was slow and often showed chlorosis on leaves. This problem was overcome by transferring microshoots to MS medium modified by increasing FeSO<sub>4</sub> (55.6 mg·L<sup>-1</sup>) and Na<sub>2</sub>EDTA (74.52 mg·L<sup>-1</sup>) salts concentration. The iron concentration had a significant effect on chlorophyll content of the leaves. Chlorophyll content was increased by increasing FeSO<sub>4</sub> and Na<sub>2</sub>EDTA salts concentration. Maximum rooting was obtained on modified MS medium supplemented with 1.0 mg l<sup>-1</sup> IBA. The *in vitro*-grown plantlets were successfully established in the field with 96% of survival. This protocol could be utilized for conservation and clonal propagation of this economically important plant.

**Key words:** Chlorosis, conservation, endangered plants, *in vitro* propagation, nodal explants, *Scrophularia takesimensis*.

## INTRODUCTION

The genus *Scrophularia* comprises about 300 species which are distributed world wide. Different members of the genus *Scrophularia* have been used as a treatment for fever, swelling, constipation, pharyngitis, neuritis, and

laryngitis (Duck and Ayensu, 1985; Qian et al., 1992). A number of iridoid glycosides, phenyl propanoids, terpenoids, and flavonoids have been isolated from *Scrophularia* species (Bhandari et al., 1997; Li et al., 2000; Kim et al., 2002; Nguyen et al., 2005). *Scrophularia takesimensis* is a rare, endemic medicinal herb species of Ullung Island in Korea (Korea Forest Service, 1997). *S. takesimensis*, commonly known as Sum-Hyun-Sum, is a perennial that is indigenous to Korea. It has been used since ancient times in traditional medicine for the treatment of fever and anti-inflammation. It is becoming endangered due to increasing human activity in the area and consequent habitat deterioration (Ahn, 2005). Hence, there is an urgent need to develop efficient techniques that allow large scale multiplication and preservation of

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**Abbreviations:** 2iP, 6-( $\gamma$ ,  $\gamma$ -dimethylallylamino) purine; BAP, 6-benzylaminopurine; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; MS, Murashige and Skoog medium; MMS, modified Murashige and Skoog medium; PGRs, plant growth regulators; TDZ, thidiazuron.

this rare species. To date there have been no reports on the propagation of this species either conventional or non-conventional methods. Tissue culture techniques have been established as a useful approach for the conservation of rare and endangered plant species (Benson et al., 2000; Latto et al., 2006; Joshi and Dhawan, 2007; Sivanesan, 2007). *In vitro* propagation method is widely used for mass multiplication and *ex situ* conservation of medicinal plants. Moreover, *in vitro* propagation of this species is important to support chemical analysis and pharmacological programs. Growth and development of plant cultures usually depends on the genotype, composition of culture medium, addition of plant growth regulators to the medium, and culture environment. The success of plant tissue culture as a means of plant propagation is greatly influenced by the nature of culture medium used. The micronutrients iron, manganese and zinc are required by all plants for proper growth and function. Some plants when lacking one or a combination of these essential micronutrients shows symptoms of yellowing or chlorosis of leaves. Chlorosis has been a problem in tissue culture of many plant species like *Chenopodium quinoa* (Radosevich and Paupardin, 1985), *Rosa hybrida* (Van der Salm et al., 1994), *Carica papaya* (Castillo et al., 1997), *Vaccinium* (Shibli et al., 1997), *Passiflora edulis* (Monteiro et al., 2000), watermelon (Thomas et al., 2000), red raspberry (Zawadzka and Orlikowska, 2006), and *Hibiscus rosasinensis* (Christensen et al., 2008). In this report, we describe the influence of plant growth regulators on axillary shoot multiplication and iron source on growth of *S. takesimensis*.

## MATERIALS AND METHODS

### Plant materials

Plants were collected during the month of May 2007 from the area of Ulunung Island, Korea. Actively growing shoots were used as explants source. Explants were washed under running tap water for 15 min and soaked in Teepol solution (0.1%, v/v) for 5 min, and then washed three times with distilled water. Thereafter, in aseptic conditions, the explants were disinfected in 70% (w/v) ethanol for 60 s and 5% (v/v) sodium hypochlorite for 15 min, followed by 5 rinses with sterile distilled water. Nodal segments were inoculated on the MS medium (Murashige and Skoog, 1962) with or without plant growth regulators (PGRs).

### Media and culture condition

The medium consisted of MS basal salts and vitamins supplemented with 3% (w/v) sucrose, and solidified with 0.8% (w/v) agar. The modification of MS medium is described elsewhere in the paper. The pH of all media was adjusted to 5.7 using 0.1 N NaOH or 0.1 N HCl before autoclaving at 121 °C for 15 min. Thidiazuron was filter sterilized and added to autoclaved medium. Other plant growth regulators were added to basal medium prior to pH adjustment and sterilization. All cultures were maintained at 25 ± 2 °C under a 16 h photoperiod with 45 µmol m<sup>-2</sup> s<sup>-1</sup> irradiance provided by cool white fluorescent light (Philips 40 W tubes).

### The effect of PGRs on axillary shoots multiplication

For shoot induction nodal segments (each with single node) were inoculated on MS medium supplemented with various cytokinins like 2iP, BAP, and TDZ, 0.5, 1.0, 2.0, 3.0 or 5.0 mg l<sup>-1</sup> alone or combination with 0.5 or 1.0 mg l<sup>-1</sup> IAA. The number of explants initiating shoot buds and the average number of shoot buds per explant were recorded after 5 weeks.

### Shoot elongation and rooting

In a preliminary experiment, microshoots of *S. takesimensis*, which had been separated from the multiple shoots were transferred to MS medium with or without IBA, and cultured for four weeks to induce the growth of shoots and roots. The results showed that microshoots grew slowly and often showed interveinal chlorosis on younger leaves. In general, iron deficient plants showed interveinal chlorosis on younger leaves. Though iron deficiency is the most common problem, manganese and zinc deficiencies may occur as well as and mimic the symptoms of iron deficiency. Whether iron or manganese or zinc is the deficient micronutrient is difficult to determine from the symptoms on the leaves. In the next experiment, MS medium was modified by doubling the usual levels of iron, manganese or zinc. The microshoots cultured on modified MS media (MMS) were evaluated initially on the capability of these elements to improve the growth of microshoots, and chlorophyll content of leaves. The results indicated that modification of MS medium with higher manganese or zinc did not improve leaf color while, increasing FeSO<sub>4</sub> and Na<sub>2</sub>EDTA content in the MS medium gave best results. To evaluate the effect of different concentrations of iron source on growth and chlorophyll content of *S. takesimensis*, microshoots (1.0 cm) were cultured on MS medium with 1.0, 1.5, 2.0, 2.5 or 3.0X FeSO<sub>4</sub> and Na<sub>2</sub>EDTA (iron chelate) for 4 weeks. Chlorophyll content was measured on young leaves with a chlorophyll meter SPAD-502 (Konica Minolta Sensing, Inc. Japan). Three measurements per leaf were taken and averaged to obtain a representative chlorophyll concentration value. For rooting, the microshoots were separated from the multiple shoots, transferred to MMS medium (FeSO<sub>4</sub> - 55.6 mg l<sup>-1</sup> and Na<sub>2</sub>EDTA - 74.52 mg l<sup>-1</sup>) supplemented with 0, 0.5, 1.0 or 2.0 mg l<sup>-1</sup> IBA. After 4 weeks shoot length, percentage of root induction, number of roots and roots length were recorded.

### Acclimatization

Healthy plantlets with well developed roots were removed from culture, washed thoroughly with running tap water and were planted in 72 cell plug trays containing a greenhouse medium (Tosilee medium, Shinan Precision Co., Jinju, Korea). Then they were placed in a mist (10 s every 10 min) chamber in the greenhouse and irrigated every alternative day with quarter-strength MS salts solution for 2 weeks. After two weeks, plants were transferred to the greenhouse bench and watered daily for 3 weeks. Acclimatized plants were transplanted into 10 cm pots containing Tosilee medium and subsequently transferred into the field. The survival rate was recorded 5 weeks after transplanting. The greenhouse was kept at 20 ± 5 °C and cooled by ventilation of an air flow of 1.3 ms<sup>-1</sup> at the same temperature. Mean, maximum, and minimum daily air temperatures and mean relative humidity were recorded during the experimental period by digital thermometers (Thermo Recorder TR-71S, T&D Corp., Matsumoto) set at 20.2, 25, 13 °C, and 66%, respectively.

### Statistical analysis

In all experiments, each treatment consisted of 25 replicates and each experiment was repeated four times. Data were statistically

**Table 1.** Effect of cytokinins on multiple shoot induction from nodal explants of *S. takesimensis*.

| Cytokinin<br>(mg. l <sup>-1</sup> ) | Shoot induction (%) |           |           | No. of shoots per explants |           |          |
|-------------------------------------|---------------------|-----------|-----------|----------------------------|-----------|----------|
|                                     | 2iP                 | BAP       | TDZ       | 2iP                        | BAP       | TDZ      |
| 0.5                                 | 69.2±0.7c           | 83.7±0.6d | 71.2±1.0a | 3.4±0.1d                   | 6.0±1.0c  | 4.4±0.5a |
| 1.0                                 | 81.7±1.0b           | 88.4±1.5c | 53.0±2.3b | 5.6±0.6c                   | 7.7±0.8b  | 3.0±0.8b |
| 2.0                                 | 84.5±1.5ab          | 93.0±0.7b | 0         | 6.4±1.2b                   | 11.4±1.2a | 0        |
| 3.0                                 | 86.0±2.0a           | 90.0±1.8a | 0         | 8.2±0.5a                   | 7.0±1.0b  | 0        |
| 5.0                                 | 42.4±1.2d           | 0         | 0         | 4.0±0.9d                   | 0         | 0        |

The data are means from three experiments with 25 replicates per treatment.

Means followed by same letters within a column are not significantly different ( $P \leq 0.05$ ).

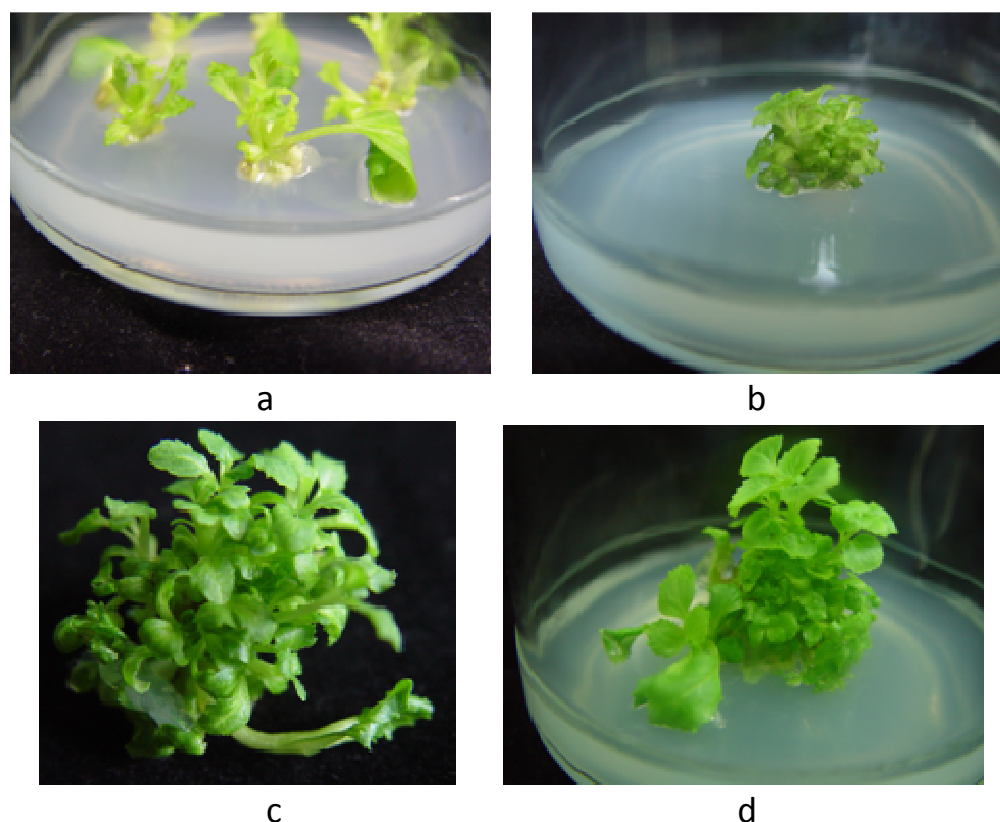
analyzed by analysis of variance (ANOVA). Significant differences between means were assessed using Duncan's multiple range test (DMRT) at 5% probability level. Data analysis was performed using SAS computer package (Release 9.1, SAS Institute Inc., NC, USA).

## RESULTS AND DISCUSSION

The described disinfection method yielded 95 - 98% aseptic explants. Nodal explants develop single shoot in growth regulator free medium. In contrast, when the shoots of the same explant were grown on culture medium containing plant growth regulators axillary shoots developed precociously, which proliferated to form secondary and tertiary shoots within 3 weeks of culture (Figure 1a and b). The formation of axillary shoot from nodal explants grown on the MS medium supplemented with different type and concentrations of cytokinins has shown in Table 1. Regenerated shoots from the nodal explant become evident after about 7 days. Analysis of variance revealed that frequency of shoot induction and number of shoots were significantly affected by the type and concentrations of cytokinins used. Nodal explants cultured on medium supplemented with 0.5 - 3.0 mg l<sup>-1</sup> BAP induced axillary shoot proliferation when compared with the control. Maximal shoot regeneration was achieved on MS medium containing 2.0 mg l<sup>-1</sup> BAP with an average of 11.2 shoots per explant (Figure 1c). An increase in BAP concentration from 0.5 to 2.0 mg l<sup>-1</sup> resulted in a significant increase in the frequency of shoot induction and an average number of shoots produced per explant, however, further increase (3.0 mg l<sup>-1</sup>) led to decrease in shoot number and browning or death (5.0 mg l<sup>-1</sup>) of the explants after 3 weeks of culture. The inhibitory effect of higher concentrations of BAP on shoot induction was reported in *Tinospora cordifolia* (Raghu et al., 2006) and *Sida cordifolia* (Sivanesan and Jeong, 2007a). Among various concentration of 2iP (0.5-5.0 mg l<sup>-1</sup>), incorporated to the MS medium 3.0 mg l<sup>-1</sup> was the most effective in inducing highest frequency (86%) with maximum shoot multiplication (8.2), whereas, the lowest frequency (42.4%) was obtained on medium supplemented with 5.0 mg l<sup>-1</sup> 2iP. Of the different concentrations of TDZ incorporated to the basal medium, profuse callus formation was observed even at low concentration (0.5 mg l<sup>-1</sup>), which

hampered shoot buds induction. Only 53% of explants produced multiple shoots with an average of 3.0 shoots per explant at 1.0 mg l<sup>-1</sup>. The explants did not developed shoots became brownish and died when the medium was supplemented with higher concentrations of TDZ. In our experiments, TDZ was less effective in axillary shoot multiplication than other cytokinins. The inhibitory effect of TDZ has been reported in *Salvia brachyodon* (Misic et al., 2006). In this study, axillary shoot proliferation in nodal explants occurred only when cytokinin was applied exogenously. However, the type and optimal concentration of cytokinin was the determining factor for axillary shoot proliferation. Among the three cytokinins tried, BAP was found to be more effective than 2iP and TDZ for axillary shoot multiplication. The application of BAP has proven extremely beneficial for multiple shoot induction in medicinal plants like *Leptadenia reticulata* (Arya et al., 2003), *Picrorhiza kurroa* (Chandra et al., 2006), *Pentaneia indicum* (Sivanesan and Jeong, 2007b), and *Sida cordifolia* (Sivanesan and Jeong, 2007a).

To evaluate the effect of combination of auxin and cytokinin on shoot development, nodal explants were culture on medium containing 0.5 or 1.0 mg l<sup>-1</sup> IAA and 1.0, 2.0 and 3.0 mg l<sup>-1</sup> BAP (Table 2). When IAA was used in combination with BAP, the frequency of shoot induction and the production of multiple shoots increased significantly after 5 weeks of culture. Among the combinations tested, the highest number of shoots (16.0) was obtained on the MS medium supplemented with 2.0 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> IAA (Figure 1d), while the lowest number of shoots (8.6) was obtained on the MS medium supplemented with BAP and IAA each at 1.0 mg l<sup>-1</sup>. Our results are in agreement with some of the previous studies, where BAP and IAA were found to be useful in multiple shoot induction from nodal explants of other medicinal plant species, e.g. *Leptadenia reticulata* (Arya et al., 2003), *Phalangium indicum* (Sivanesan and Jeong, 2007b), and *Withania somnifera* (Sivanesan, 2007, Sivanesan and Murugesan, 2008). After harvesting the microshoots, the mother explants were subcultured on same fresh medium (2.0 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> IAA) after every 5 weeks resulted in high frequency of regeneration. An average of 2-fold multiplication was achieved within 15 weeks of initial culture which could be maintain-



**Figure 1.** Axillary shoot multiplication from nodal explants of *S. takesimensis*. a) shoot induction on MS medium with 2.0 mg l<sup>-1</sup> BAP after 1 week, b) multiple shoot induction on MS medium with 2.0 mg l<sup>-1</sup> BAP after 3 weeks, c) multiple shoot induction on MS medium with 2.0 mg l<sup>-1</sup> BAP after 5 weeks, d) multiple shoot induction on MS medium with 2.0 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> IAA after 5 weeks.

**Table 2.** Effect of different concentrations and combination of BAP and IAA on multiple shoot induction from nodal explants of *S. takesimensis*.

| PGR (mg. l <sup>-1</sup> ) |     | Shoot induction (%) | No. of shoots per explants |
|----------------------------|-----|---------------------|----------------------------|
| BAP                        | IAA |                     |                            |
| 1.0                        | 0.5 | 92.0±1.6e           | 9.4±0.6d                   |
| 2.0                        | 0.5 | 96.2±1.0c           | 13.2±0.4b                  |
| 3.0                        | 0.5 | 94.8±1.5d           | 12.0±0.1c                  |
| 1.0                        | 1.0 | 98.4±0.1b           | 8.2±0.6e                   |
| 2.0                        | 1.0 | 100a                | 16.0±0.5a                  |
| 3.0                        | 1.0 | 96.0±2.0c           | 12.4±1.0bc                 |

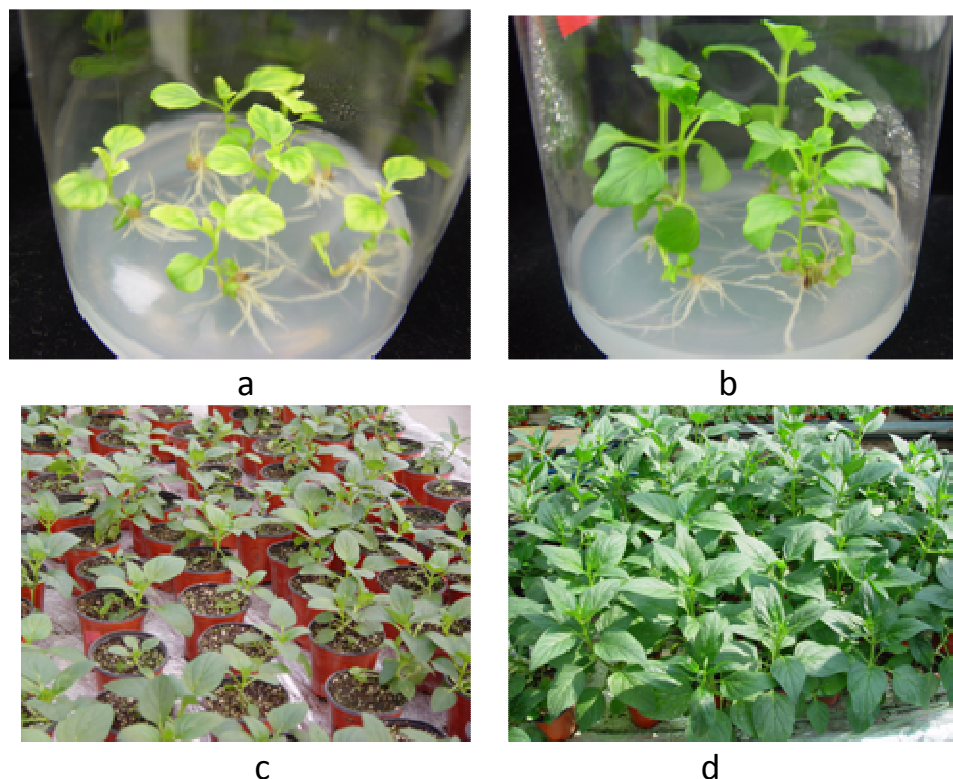
The data are means from three experiments with 25 replicates per treatment.

Means followed by same letters within a column are not significantly different ( $P \leq 0.05$ ).

ed for longer periods without any loss in the morphogenetic potential.

Shoot growth and root initiation were visible within a week following the transfer of microshoots to auxin free MS medium and 68% of shoots produced an average of 4.6 roots, each measuring 2.6 cm over a period of 4

weeks. However, microshoots were cultured on MS medium grew slowly, and often showed chlorosis in younger leaves at 14 days and it was more severe at 21 days (Figure 2a). Chlorosis was overcome by doubling the concentration of iron chelate ( $\text{FeSO}_4$  and  $\text{Na}_2\text{EDTA}$ ) in the medium (Figure 2b). Similar results have been reported in other plant species, *Rhododendron* (Anderson, 1984), *R. hybrida* (Van der Salm et al., 1994), and *Delphinium cardinale* (Ohki and Sawaki, 1999). Plants growing on a sugar supplemented medium *in vitro* produce only a small amount of their carbohydrate requirement through  $\text{CO}_2$  fixation when they are taken from culture they have to change to fully autotrophic nutrition. Chlorophyll, the green pigment is required for photosynthesis in plants. The reduction of chlorophyll content would reduce the photosynthesis by decreasing light absorption. Therefore, chlorosis can affect plant growth and survival rate during acclimatization. Thus, plantlets with higher chlorophyll content might have a higher chance of survival and better growth and development during acclimatization due to higher carbohydrate reserve and photosynthetic competence (Christensen et al., 2008). Growth and chlorophyll content of some species may even be promoted by increasing the level of iron source



**Figure 2.** Shoot elongation, rooting and acclimatization of *S. takesimensis*. a) microshoots cultured on MS medium with 1.0 mg l<sup>-1</sup> IBA after 4 weeks, b) microshoots cultured on MMS medium with 1.0 mg l<sup>-1</sup> IBA after 4 weeks, c & d) acclimatized plants grown in a greenhouse.

above that recommended by Murashige and Skoog (1962). In this study, the iron concentration had a significant effect on chlorophyll content of the leaves. Leaf chlorophyll content was increased with increasing iron concentration in the medium. Iron is required for the formation of amino laevulinic acid and protoporphyrinogen, which are early and late precursors of chlorophyll, respectively and is a component of many key metallo-proteins (Merchant and Dreyfuss, 1998). When the iron concentration was doubled, chlorophyll content was three times higher as compared with the control (Table 3), however, further increase led to decrease in chlorophyll content but no chlorosis was observed. Shoot growth was significantly affected by iron level. The mean shoot lengths were 3.5, 4.3, 4.8, 4.0, and 3.2 cm at 1.0, 1.5, 2.0, 2.5 and 3.0X iron chelate, respectively. Similar results have been reported in other plant species like Watermelon (Thomas et al., 2000), and *H. rosasinensis* (Christensen et al., 2008). The percentage of rooting response, root number, root length and shoot length were varied significantly with different concentrations of IBA (Table 3). The best rooting response (100%) was obtained on MMS medium supplemented with 1.0 mg·L<sup>-1</sup> IBA with an average of 10.2 roots per shoot and root length 4.2 cm over a period of 4 weeks (Figure 2b). The addition of IBA favored rooting in other medicinal plants

**Table 3.** Effect of iron concentration on growth and chlorophyll content of *S. takesimensis*.

| Iron chelate (X) | Chlorophyll content (SPAD units) | Shoot length (cm) |
|------------------|----------------------------------|-------------------|
| 1.0              | 14.96±1.5e                       | 3.5±0.31d         |
| 1.5              | 36.57±5.4c                       | 4.3±0.07b         |
| 2.0              | 43.29±4.3a                       | 4.8±0.09a         |
| 2.5              | 39.04±3.3b                       | 4.0±0.07c         |
| 3.0              | 34.41±1.4d                       | 3.2±0.11e         |

1X = FeSO<sub>4</sub> (27.8 mg·L<sup>-1</sup>) and Na<sub>2</sub>EDTA (37.26 mg·L<sup>-1</sup>).

The data are means from three experiments with 25 replicates per treatment.

Means followed by same letters within a column are not significantly different ( $P \leq 0.05$ ).

like *P. kurrooa* (Chandra et al., 2006), *P. indicum* (Sivanesan and Jeong, 2007b), *S. cordifolia* (Sivanesan and Jeong, 2007a), and *W. somnifera* (Sivanesan, 2007). Low concentrations of IBA favored longer shoots, but the percentage of root induction and number of roots per shoot decreased while high concentration of IBA in the MMS medium resulted in inhibition of shoot growth and profuse callusing rather than roots from the base of the shoots.

**Table 4.** Effect of IBA on rooting and shoot growth of *S. takesimensis*.

| IBA (mg. l <sup>-1</sup> ) | Root induction (%) | Number of roots per shoot | Root length (cm) | Shoot length (cm) |
|----------------------------|--------------------|---------------------------|------------------|-------------------|
| 0 (MS)                     | 68.0±1.6c          | 4.6±0.22d                 | 2.6±0.08c        | 3.5±0.31c         |
| 0 (MMS)                    | 70.4±1.5d          | 6.0±0.38c                 | 3.2±0.10b        | 4.8±0.09b         |
| 0.5                        | 87.6±1.0b          | 7.4±0.52b                 | 3.6±0.08b        | 5.7±0.16a         |
| 1.0                        | 100a               | 10.2±1.0a                 | 4.2±0.11a        | 5.1±0.12ab        |
| 2.0                        | 0                  | 0                         | 0                | 1.4±0.10d         |

The data are means from three experiments with 25 replicates per treatment.

Means followed by same letters within a column are not significantly different ( $P \leq 0.05$ ).

Healthy plantlets with well developed roots were planted in 72 cell plug trays containing a greenhouse medium. They were irrigated with quarter-strength MS salts (Tosilee medium) and acclimatized in a mist (10 s every 10 min) chamber in the greenhouse for two weeks, and the survival rate was 100% (Figure 2c and d). Micro-propagation protocol is successful only when the rate of survival of the *in vitro*-grown plantlet is very high after transplantation. The acclimatized plants were reintroduced into their natural habitat and the survival rate was 96%, 5 weeks after transfer to the field. The plantlets thus developed from nodal explants were uniform and identical to donor plants with respect to morphological and growth characteristics.

## Conclusion

This is the first report describing reproducible protocol for micro-propagating *S. takesimensis* using nodal explants. MS medium supplemented with 2.0 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> IAA is the most effective medium for axillary shoot multiplication. Chlorosis can be overcome by doubling the usual levels of FeSO<sub>4</sub> and Na<sub>2</sub>EDTA in the medium. Maximum rooting was obtained on MMS medium fortified with 1.0 mg l<sup>-1</sup> IBA. The *in vitro*-grown plantlets were successfully established in the field with 96% survival. This protocol could be utilized for conservation and clonal propagation as well as chemical analysis of this economically important plant.

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