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MICROPROPAGATION AND GREENHOUSE CULTIVATION OF SCROPHULARIA TAKESIMENSIS NAKAI, A RARE ENDEMIC MEDICINAL PLANT

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Abstract

A protocol was developed for direct shoot regeneration of *Scrophularia takesimensis* using shoot tip explants. The explants were excised from mature field grown plants and cultured on Murashige and Skoog (MS) medium with different concentrations of 2-isopentyl adenine (2-iP), 6-benzyl adenine (BA) or thidiazuron (TDZ). Among the three cytokinins studied, BA was found to be the most effective cytokinin for multiple shoot induction. Addition of auxin to the shoot regeneration medium significantly enhanced the percentage of shoot induction and number of shoots per explant. The greatest percentage of shoot induction was achieved when shoot tip explants were cultured on MS medium supplemented with 3.0 mg L^{-1} BA and 1.0 mg L^{-1} indole-3-acetic acid (IAA) with an average of 13 shoots per explant. The regenerated shoots were transferred to modified MS medium supplemented with 3% (w/v) sucrose, 1.0 mg L^{-1} indole-3-butyric acid (IBA), and solidified with 0.8% (w/v) agar for four weeks to induce the growth of shoots and roots. Plantlets were transferred to the greenhouse with 100% survival rate. High performance liquid chromatography analysis detected the presence of harpagoside in greenhouse-grown plants organs which were established from *in vitro* culture. The content of harpagoside was high in fruit followed by leaf, root and stem. This protocol could be utilized for conservation and clonal propagation and chemical analysis of this economically important plant.

Introduction

The genus *Scrophularia* includes approximately 312 species of annual, biennials or perennial herbs, which are distributed worldwide. Many species in this genus have been used in the traditional medicine since antiquity. 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). Iridoid glycosides are considered to be the main bioactive components, with antioxidant, antiinflammatory, antimicrobial, antidiabitic, and neuroprotective activities (Ahmed *et al*., 2003; Kim *et al*., 2003; Jeong *et al*., 2008; Li *et al*., 2009). Harpagoside, the major bioactive iridoid glycoside, has been shown to posses antioxidative, antiinflammatory and neuroprotective activities (Kim *et al*., 2002; Ahmed *et al*., 2003; Jeong *et al*., 2008). *Scrophularia takesimensis* Nakai is a critically endangered medicinal herb species endemic to Ulleung Island, a small volcanic island located in the East Sea approximately 150 km from the mainland of Korea (Sun & Stuessy, 1998). It has been used since ancient times in traditional medicine for the treatment of fever and anti-inflammation. It grows to a height of 1m in the coastal area. The glabrous stems are square. Leaves are opposite with serrated margins. The flower blooms between June and July. The construction of coastal routes and small harbors has become the most serious threat to its conservation, and many populations have already disappeared from that island (Lim *et al*., 2008). Hence, there is an urgent need to develop efficient techniques that allow large scale multiplication and preservation of this rare species. Tissue culture techniques have been established as a useful approach for the conservation of rare and endangered plant species (Abbas & Qaiser, 2010; Abbas *et al*., 2010; Rout *et al.*, 2011). We previously reported direct shoot regeneration from nodal explants of *S. takesimensis* (Sivanesan *et al*., 2008). However, there is no report available using shoot tip explants derived from mature plants. The objectives of this study were to determine the effect of plant growth regulators on axillary shoot multiplication from shoot tip explants of *S. takesimensis*, to evaluate the growth of micropropagated plantlets in the greenhouse, and to assess harpagoside content in the greenhouse-grown plants.

Materials and Methods

Plant materials: Plants were collected from the area of Ulleung Island, Korea. Actively growing shoots were used as explants source. Explants were washed under running tap water for 30 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% (v/v) ethanol for 60 sec, 1.5% (v/v) sodium hypochlorite for 15 min, and 0.1% (w/v) mercuric chloride for 5 min. Each treatment was followed by 4-5 rinses with sterile distilled water.

Media and culture condition: The medium consisted of Murashige and Skoog (1962) basal salts and vitamins (MS) 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^{-2} s^{-1} irradiance provided by cool white fluorescent light (PHILIPS 40 W tubes).

The effect of plant growth regulators (PGRs) on shoot induction: For shoot induction shoot tip explants were inoculated on the MS medium supplemented with various cytokinins like 2-iP, BA, and TDZ, 0.5, 1.0, 2.0, 3.0 or 5.0 mg L^{-1} alone or combination with 0.5 or 1.0 mg L^{-1} IAA. The number of explants initiating shoot buds and the average number of shoot buds per explant were recorded after 5 weeks.

Shoot elongation, rooting and acclimatization: The shoot buds were transferred to modified MS medium ($FeSO_4$ - 55.6 mg l^{-1} and Na_2EDTA - 74.52 mg L^{-1}) supplemented with 1.0 mg L^{-1} IBA and cultured for 4 weeks to induce the growth of shoots and roots (Sivanesan *et al*., 2008). Plantlets with well-developed roots were removed from culture, washed thoroughly with sterile water, planted out in acclimatization boxes containing a commercial medium (Tosilee medium, Shinan Grow, Jinju, Korea), irrigated every alternative days with quarter strength MS salts solution, and maintained in a greenhouse. After 3 weeks, acclimatized plants were transplanted into 10 cm pots containing a Tosilee medium and a nutrient solution was supplied uniformly twice a day (Sivanesan *et al*., 2010). The plants were grown in a greenhouse at Gyeongsang National University, Jinju, under a normal day-light condition with night/day set temperatures of 19/27°C and 60-70% RH.

Extraction and quantification of harpagoside: Plant materials (greenhouse-grown plants) were dried in a hot air oven at 50°C for 3 days, powdered and (100 mg) extracted with (40 mL) methanol at 40°C. The extract was filtered through a Whatman No. 1 filter paper, and evaporated under reduced pressure. The residue was dissolved in HPLC-grade methanol and filtered through a membrane filter (0.2 µm pore size). The HPLC analysis was performed on a Waters high performance liquid chromatography equipped with a ODS HYPERSIL (4.6 mm i.d. x 250 mm, 5 µm) column, and a UV detector operating at a wavelength of 280nm. The mobile phase (methanol: water, 50:50) was pumped at a flow rate of 1.0 mL per min. The quantitative analysis of the harpagoside, based on the UV detection, was performed using an external standard. A standard calibration curve was plotted by using different concentrations of harpagoside (Sigma-Aldrich, Germany).

Statistical analysis: The experiments were set up in a completely randomized design and repeated thrice. All data were analyzed by analysis of variance (ANOVA) followed by Duncan multiple range test. Data analysis was performed using SAS computer package (Release 9.1, SAS Institute Inc., NC, USA).

Results and Discussion

The surface sterilization and explant preparation procedures described above yielded 96% aseptic shoot tips. The formation of axillary shoot from shoot tip explants grown on the MS medium supplemented with different type and concentrations of cytokinins is shown in Table 1. Lack of cytokinin mostly produced a single shoot, but the addition of cytokinin to the MS medium significantly stimulated shoot multiplication. Therefore, inclusion of cytokinin was essential to induce bud break in shoot tip explants. Analysis of variance revealed that frequency of shoot induction and number of shoots were significantly affected by the type and concentrations of cytokinins used. The explants shoot initiation started after 7, 12, or 16 days of culture when the MS medium was supplemented with TDZ, BA or 2-iP, respectively. The stimulating effect of TDZ on shoot induction has been reported in many plant species (Lu, 1993; Sajid & Aftab, 2009; Sivanesan *et al*., 2011). Of the different concentrations of TDZ incorporated to the MS medium, 0.5 mg L^{-1} proved to be optimum for multiple shoot induction. However, higher concentrations of TDZ reduced or completely inhibited shoot induction and number of shoots per explant (Table 1). The inhibitory effect of TDZ may due to its high cytokinin activity (Huetteman & Preece, 1993).When the explants were cultured on the MS medium supplemented with 2iP (0.5-3.0 mg L^{-1}), the multiple shoot induction efficiency increased from 62.0-77.0%. Number of shoot buds per explant increased with increasing the concentration of 2-iP in the culture medium. Maximal shoot regeneration was achieved when the medium containing 3.0 mg L^{-1} 2-iP with an average of 6.1 shoots per explant. Increasing 2-iP concentration above 3.0 mg L^{-1} had a negative effect on the frequency of shoot induction and number of shoots per explant (Table 1). When shoot tip explants cultured on the MS medium with 0.5-3.0 mg L^{-1} BA induced multiple shoots as compared with the control. The highest average number of shoots (7.2) was obtained on the MS medium containing 2.0 mg L^{-1} BA and the corresponding percentage of shoot induction was 71.8%. An increase in BA concentration from 0.5 to 2.0 mg L^{-1} 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^{-1}) led to decrease in shoot number and browning or death (5.0 mg L^{-1}) of the explants after 5 weeks of culture (Table 1). The inhibitory effect of higher concentrations of BA on shoot induction has also been reported in other plant species such as *Simmondsia chinensis* (Agrawal *et al*., 2002), *Cassia siamea* (Parveen *et al*., 2010) and *Cypripedium formosanum* (Lee, 2010). Of the three cytokinins tested, BA was found to be most effective than 2-iP and TDZ for multiple shoot induction from shoot tip explants of *S. takesimensis*. The application of BA has proven extremely beneficial for shoot multiplication in many medicinal plant species such as *Stevia rebaudiana* (Rafiq *et al*., 2007), *Cadaba heterotricha* (Abbas & Qaiser, 2010), *Anethum graveolens* (Jana & Shekhawat, 2011), balck pepper (Ahmad *et al*., 2011) and *Withania somnifera* (Rout *et al*., 2011).

Table 1. Effect of cytokinins on shoot induction from shoot tip explants of *S. takesimensis* after 5 weeks of culture.

Conc. (mg L^{-1})	Shoot induction (%)			No. of shoots per explant		
	2iP	BA	TDZ	2iP	BA	TDZ
0.5	62.0d	61.7c	68.0a	2.7d	3.0c	5.0a
1.0	69.1c	65.0b	60.4b	3.6c	4.7b	3.2b
2.0	74.8b	71.8a	0	5.2b	7.2a	0
3.0	77.2a	59.7d	0	6.1a	3.8c	0
5.0	54.6e	0	0	3.0c	0	0

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

Although cytokinins are very effective in promoting axillary shoot multiplication, an auxin is almost invariably required to promote the initial growth of meristem and shoot tip explants. Thus, a combination of an auxin and a cytokinin is frequently used to obtain high frequency shoot induction. To evaluate the effect of combination of auxin and cytokinin on shoot multiplication, shoot tip explants were cultured on medium containing 0.5 or 1.0 mg L^{-1} IAA and 1.0, 2.0 and 3.0 mg L^{-1} BA (Table 2). When BA was used in combination with IAA, the percentage of shoot induction and the average number of shoots per explants increased significantly as compared to BA alone. Among the combinations tested, the greatest average number of shoots (13.0) was obtained on the MS medium supplemented with 2.0 mg L^{-1} BA and 1.0 mg L^{-1} IAA (Fig. 1A), while the lowest mean number of shoots (7.6) was obtained on the MS medium supplemented with 1.0 mg L^{-1} BA and 0.5 mg L^{-1} IAA . This is in agreement with other researchers (Abbas *et al*., 2010; Jana & Shekhawat, 2011; Rout *et al.*, 2011) where a synergistic effect of auxin and cytokinin has been reported in other plant species. The regenerated shoots were transferred to modified MS medium containing 1.0 mg L^{-1} IBA for shoot elongation and rooting. After 4 weeks of culture 100% root induction was achieved on the above medium (Fig. 1B). Plantlets hardened in the acclimatization box benefited from the high humidity and exhibited 100% survival (Fig. 1C). Micropropagation protocol is successful only when the rate of survival of the *in vitro*-grown plantlet is very high after transplantation. After 3 weeks, acclimatized plants were transplanted into 10 cm pots containing a Tosilee medium and a nutrient solution was supplied uniformly twice a day and the survival rate was also 100% after a month (Fig. 1D). The biological activities and chemical composition of *S. takesimensis* has not been studied because it is protected by the regulations of the Korea Forest Service as a rare plant. Thus, cultivation of this endemic plant species permits production of uniform and high quality raw material for screening biological activities and chemical analysis. A mean plant height, stem diameter and length, number of branches, inflorescence number and length, chlorophyll content, fresh and dry weights of shoot and roots were presented in Table 3. The regenerated plantlets were uniform and identical to donor plants with respect to morphological and growth characteristics after 6 months of cultivation (Fig. 1D-F). High performance liquid chromatography analysis detected the presence of harpagoside in greenhouse-grown plants organs which were established from *in vitro* culture; however, other peaks on the chromatogram may be identified in the future (Fig. 2). The content of harpagoside was high in fruit followed by leaf, root and stem (Fig. 3).

Table 2. Effect of different concentrations and combination of BA and IAA on shoot induction from shoot tip explants of *S. takesimensis* after 5 weeks of culture.

PGR (mg L^{-1})		Shoot induction (%)	No. of shoots per explant
BA	IAA		
1.0	0.5	84.4d	7.6c
2.0	0.5	91.2c	9.4bc
3.0	0.5	96.4b	9.8b
1.0	1.0	95.6b	8.0c
2.0	1.0	100a	13.0a
3.0	1.0	100a	10.2b

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

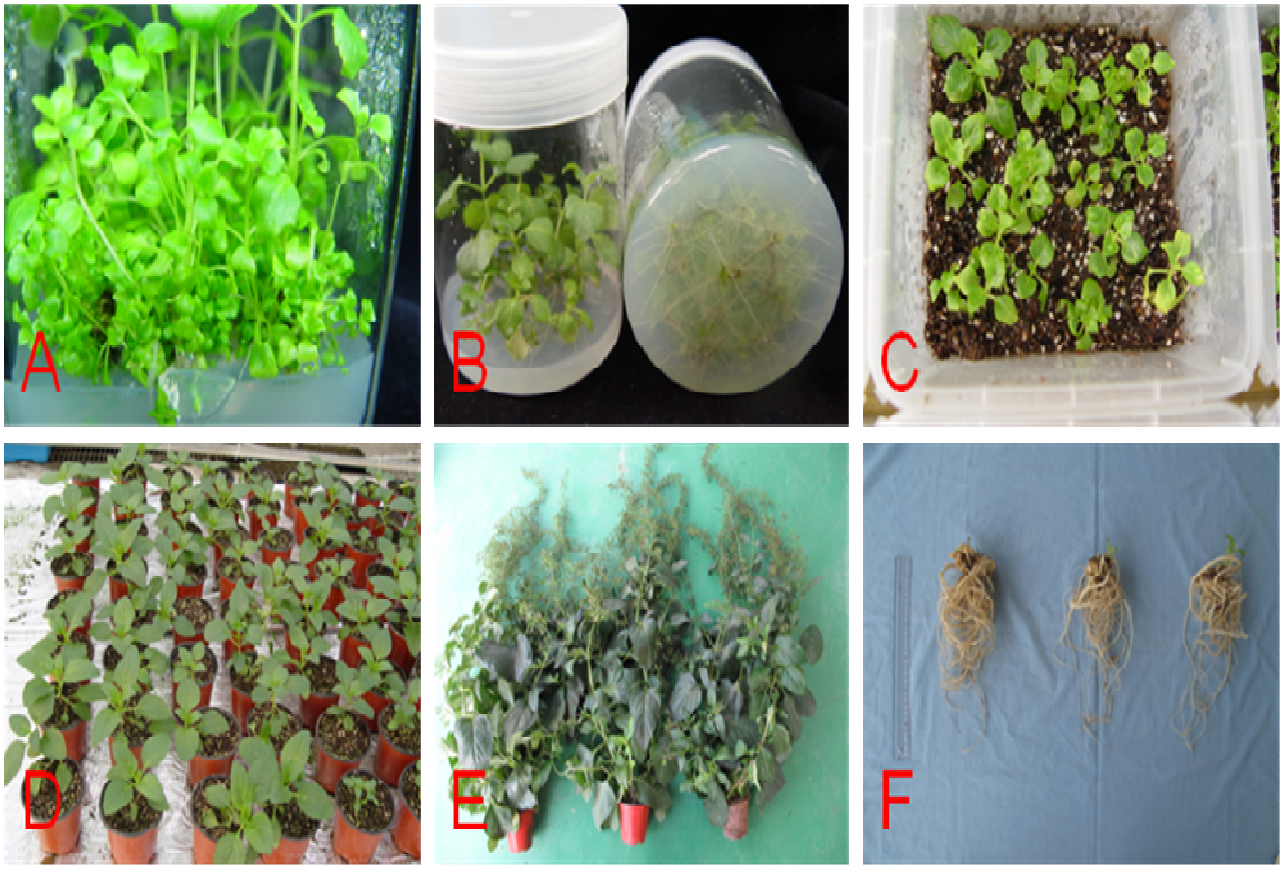

Fig. 1. Micropropagation of *Scrophularia takesimensis*. A) Multiple shoots induction on MS medium with 2.0 mg L^{-1} BA + 1.0 mg L^{-1} IAA, B) Shoot elongation and rooting of microshoots on modified MS medium supplemented with 1.0 mg L^{-1} IBA, C) Plantlets transferred into the acclimatization box, D & E) Acclimatized plantlets cultivated under greenhouse conditions, and F) Harvested roots after 6 months of cultivation.

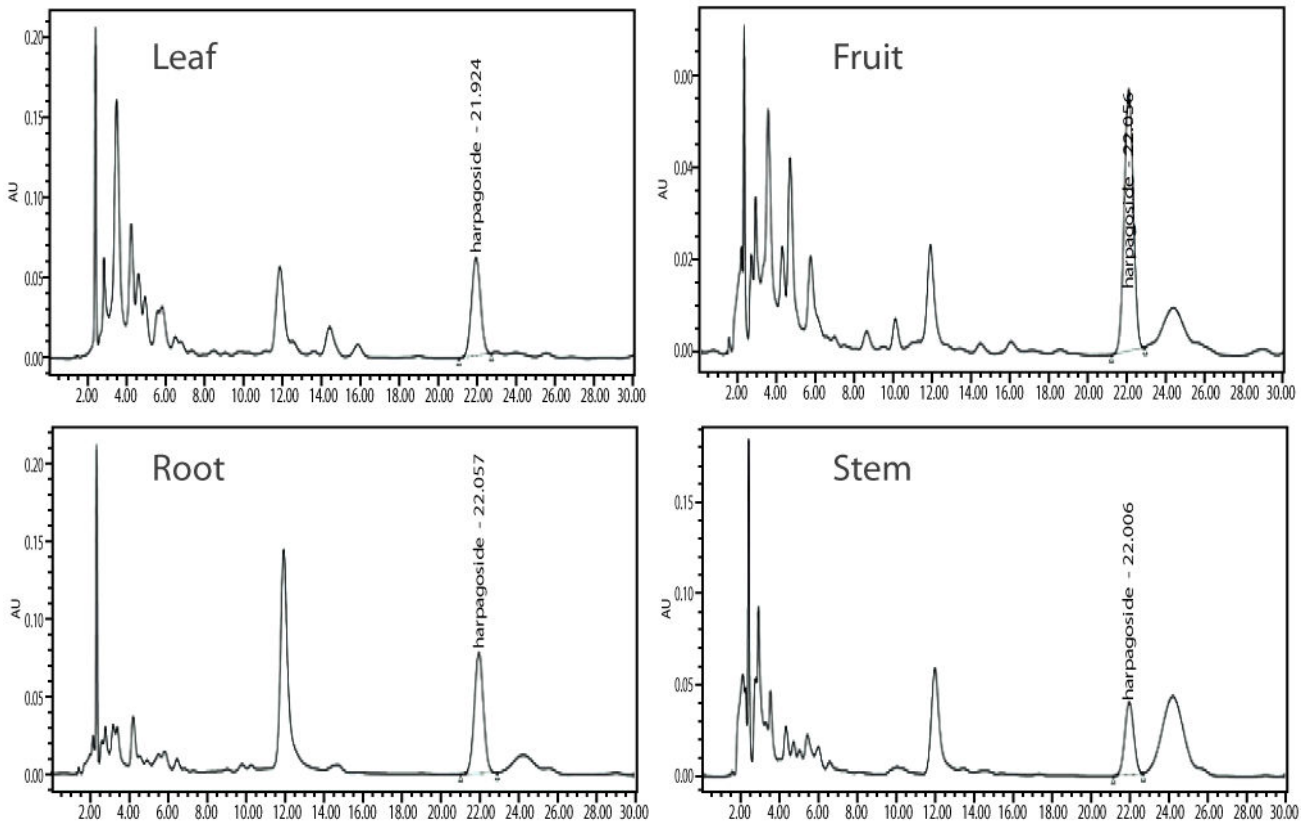

Fig. 2. HPLC chromatograms of *S. takesimensis*.

Table 3. Growth traits measured after 6 months of greenhouse cultivation of *S. takesimensis*.

Plant height (cm)	Stem length (cm)	Stem diameter (mm)	No. of branches	No. of inflorescences	Inflorescence length (cm)	Chlorophyll content (SPAD)	Fresh weight (g)		Dry weight (g)	
							Shoot	Root	Shoot	Root
125.4±7.7	52.6±2.1	10.4±0.7	4.3±1.2	23.7±2.1	78.5±5.0	52.1±2.2	669.7±19.5	104.3±7.6	152.6±5.3	18.5±3.7

Values represent mean ± standard deviation

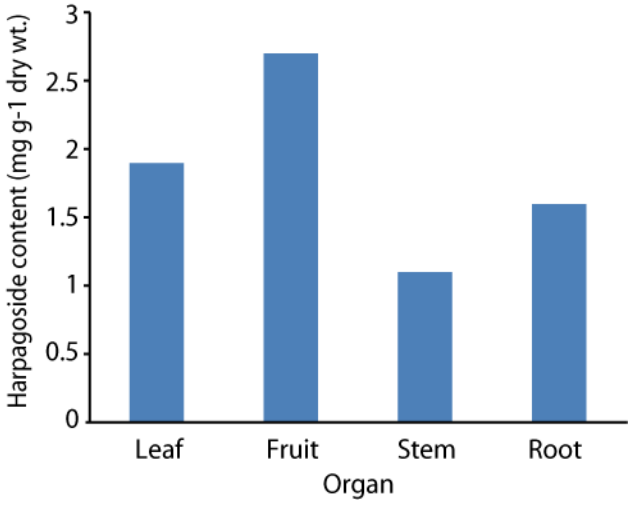

Fig. 3. Harpagoside content in different organs of micropropagated *S. takesimensis* 6 months after cultivation.

Conclusion

In this study, protocols for cultivation of *S. takesimensis* have successfully been standardized. The MS medium supplemented with 2.0 mg L^{-1} BA and 1.0 mg L^{-1} IAA is most effective medium for axillary shoot multiplication. The regenerated shoots were rooted best on modified MS medium containing 1.0 mg L^{-1} IBA. The rooted plants were acclimatized and cultivated in the greenhouse. High performance liquid chromatography analysis detected the presence of harpagoside in greenhouse-grown plants organs. Therefore, this protocol could be utilized for conservation and clonal propagation and chemical analysis of this economically important plant. Such reports have already been published by Hussain *et al*., 2011 and Abbasi *et al*., 2011.

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