JEE Journal of Ecological Engineering

Journal of Ecological Engineering, 2025, 26(10), 1–10
https://doi.org/10.12911/22998993/204543
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Received: 2025.03.27
Accepted: 2025.07.15
Published: 2025.07.22

A micropropagation protocol for American skullcap (*Scutellaria lateriflora* L.) – a plant rarely found in Europe

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ABSTRACT

The wide range of applications of *Scutellaria lateriflora* L. encourages research on the effective regeneration of this species *in vitro*. In order to introduce this species in Poland, an alternative method of obtaining plants is morphogenesis from existing meristematic tissues. The aim of the study was to obtain numerous side shoots from single-node shoot fragments. *In vitro* culture was performed on Murashige and Skoog (MS) medium, and plant growth regulators (PGRs) belonging to the auxin and cytokinin groups were used: NAA (naphthyl-1-acetic acid), BAP (6-benzylaminopurine). The obtained plants were analyzed for genetic stability using SCoT markers. The use of 2 mg·dm^{-3} BAP in MS medium proved to be the best in terms of obtaining the highest number of axillary shoots per 1 explant (9.20). In this variant the multiplication factor (the number of axillary shoots obtained in relation to the number of rooted plants) was 15.3. Induction of rhizogenesis was achieved on all planted explants, both in the control variant (0 PGRs) and in the variant in which the medium was enriched with IAA (indolyl-3-acetic acid) in the amount of 0.5 mg·dm^{-3}. In the presented experiment, SCoT molecular marker revealed a high degree of genetic fidelity of micropropagated of plants.

Keywords: American skullcap, axillary shoots, micropropagation, molecular marker, plant growth regulators, rooting, somaclonal variation.

INTRODUCTION

American skullcap (*Scutellaria lateriflora* L.) belongs to the group of plants of the genus *Scutellaria* L., which includes about 460 species. The name of the genus in Latin means a shield, the shape of which resembles the cover of the calyx of thyroid flowers (Hryć and Zgórka, 2018). In Poland, there are three species belonging to the genus *Scutellaria:* common skullcap (*S. galericulata*), spear-leaved skullcap (*S. hastifolia*) and Somerset skullcap (*S. altissima*). The herbal raw material of *S. lateriflora* is the herb or root. The health-promoting properties of this species were first discovered by Indians living in North America. American skullcap is widely used for medicinal purposes in North America. It is also available in the UK and Australia. They can be purchased in pharmacies, herbal shops and online in various forms of tablets, capsules, teas and tinctures (Kwiecień et al., 2019). Flavonoids from the flavones group specific to the genus *Scutellaria* have been isolated from the herb or root of this species, including: baicalin, baicalein, wogonin, wogonoside and scutellarin. These compounds exhibit anti-inflammatory, antiviral, cytotoxic and anticancer activity, and also have hepatoprotective and neuroprotective effect (Lohani et al., 2013; Wu et al., 2016; Oche et al., 2016; Wilczańska-Barska et al., 2020; Zehravi et al., 2022; Xie et al., 2024). The presence of other

chemical compounds that can be used for therapeutic purposes has also been confirmed in the herb. These include essential oils (calamenene, caryophyllene and limonene), phenolic acids, diterpenoids, coumarins, waxes, amino acids, which make it valuable as a herbal raw material (Kwiecień et al., 2019; Lawson et al., 2021). American skullcap was originally used to treat various diseases of the nervous system. The bioactive substances contained in the tissues of this species effectively relieve states of nervous tension, have an antianxiety and anticonvulsive effect (Brock et al., 2014). The herb of *S. lateriflora* is successfully used in the treatment of smooth muscle spasms and also as a stimulant for the urinary, digestive, respiratory and reproductive systems (Lawson et al., 2021; Brock et al., 2014; Kawka et al., 2017; Tuan et al., 2018; Kwiecień et al., 2022; Ullah et al., 2023). The wide range of possible uses of American skullcap encourages research to achieve effective regeneration of this species under controlled conditions. However, there is a lack of information in the literature on the micropropagation of American skullcap. Using micropropagation it is possible to quickly obtain a large number of daughter plants, balanced in terms of phenotype and genotype (Kulus and Woźny, 2020; Magyar-Tábori et al., 2021; Baby et al., 2024). Micropropagation is a technique that is an alternative to conventional plant propagation. *In vitro* culture conditions can cause the appearance of somaclonal variability among regenerated plants (Krishna et al., 2016). One of the ways of obtaining plants is morphogenesis from existing meristematic tissues. Among the various methods of micropropagation, the induction of axillary bud growth is the most common. The types of explants used in this technique are: shoot tips, single axillary buds, shoot node sections, shoot fragments with a leaf and an active axillary bud (Baby et al., 2024). The research was aimed at obtaining *S. lateriflora* plants by direct organogenesis from explants of single-node shoot fragments obtained from sterile this species; and to assess of genetic stability of *S. lateriflora* plants obtained by micropropagation using SCoT markers.

MATERIALS AND METHODS

Plant material

The seeds of the American skullcap (*S. lateriflora*) were collected in August 2022 from the greenhouse belonging to Bydgoszcz University of Sciences and Technology (Poland) (53°07'15.6"N 18°00'23.1"E). From the collection of 20 plants, seeds were manually isolated and subjected to chemical sterilization.

Seed sterilization and germination induction

The research was conducted in March 2023. The plant material for the study was plants of American skullcap. The experiment was carried out in March 2023. The initial explant was the seeds. In the experimental part, ½ Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) (Sigma-Aldrich, Burlington, MA, USA) was used as the basic medium for plant multiplication *in vitro*, which, in addition to the basic macro and microelements, contained: inositol (100 $mg \cdot dm^{-3}$), thiamine (0.4 $mg\ dm^{-3}$) and sucrose (30 $g \cdot dm^{-3}$) and was solidified with agar (8 $g \cdot dm^{-3}$) (Biomaxima, Lublin, Poland). The medium was enriched with GA_3 (gibberellic acid) (Sigma-Aldrich, Burlington, MA, USA) in the concentration of 2 $mg \cdot dm^{-3}$. The pH of the prepared medium was 5.8. The medium was poured into test tubes and sterilized in an autoclave (20 min., 121 °C, 0.5 atm.). The seed disinfection process took place in 70% ethanol (C_2H_5OH) for 60 seconds and 2% sodium hypochlorite (NaClO) for 11 minutes. The germination process took place under controlled conditions, in a growth room at a temperature of 24 ± 2 °C, with 16 hours of light (PILA fluorescent light with a quantum intensity of 40 $\mu mol\ m^{-2}\ s^{-1}$) and 8 hours of darkness (Figure 1).

Shoot propagation

Seedlings obtained from seeds under sterile conditions were cut into single-node shoot fragments containing active axillary buds. The explants were passaged (I passage) individually into flasks on PGRs free MS medium (0 PGRs) to induce the development of axillary buds. In the second passage, explants were isolated from developed side shoots and inoculated onto MS medium supplemented with PGRs. The control was variant (A), which used 0 PGRs medium. The effects of the most frequently used PGRs for micropropagation were compared, namely: 1-naphthylacetic acid (NAA) and 6-benzylaminopurine (BAP) (Sigma-Aldrich, Burlington, MA, USA). in various combinations and quantities

Figure 1. American skullcap (*S. lateriflora*) seedling in *in vitro* culture

(B-E): 0 PGRs (A); 1.0 mg·dm^{-3} BAP (B); 2.0 mg·dm^{-3} BAP (C); 1.0 mg·dm^{-3} BAP + 0.5 mg·dm^{-3} NAA (D); 2.0 mg·dm^{-3} BAP + 1.0 mg·dm^{-3} NAA (E). Subsequent phases of the *in vitro* culture of *S. lateriflora* were carried out in a growth room, under previously established lighting and temperature conditions.

Induction of root formation and acclimatization of plants to in vivo conditions

American skullcap shoots obtained at the induction stage were transferred to rooting medium after 5 weeks of growth. In the rooting process, different variants of MS media were used, one of which contained PGR and the other one was without PGRs. The most commonly used auxin, IAA (indolyl-3-acetic acid) (Sigma-Aldrich, Burlington, MA, USA), was used for this purpose in the amount of 0.5 mg.dm^{-3}. The rhizogenesis process took place under controlled temperature and lighting conditions in a growth room. After 4 weeks, the microcuttings of the American skullcap were planted into multiplate containing horticultural peat. A total of 90 plants were planted. The plants were watered and sprinkled, and protected against excessive water evaporation by covering the multiplate with food foil. After 14 days of growth, the acclimatized plants were planted individually into pots containing peat and placed in a greenhouse (Figure 2a, Figure 2b).

Statistical analysis

The obtained numerical results concerning the effect of PGRs on the number and length of shoots and roots of *in vitro* American skullcap plants (*S. lateriflora*) were subjected to analysis of variance. One-way analysis of variance (ANOVA) was performed using the Statistica® 13.1 package for calculations. The significance of differences (LSD) was assessed using Tukey's single confidence intervals for the significance level of $p \leq 0.05$. The tables also provide arithmetic mean values ± SD (standard deviation).

Molecular assays

Genomic DNA was isolated from plants of American skullcap obtained in every variant of MS media: A-D and a seed-derived plants. DNA was isolated from two randomly selected plants of each variant of experiment. The isolation was performed from 100 mg of leaf tissue with the GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Scientific, Waltham, MA, USA). For the PRC reaction the Taq polymerase (A&A Biotechnology, Gdansk, Poland) and 15 SCoT primers (Laboratory of DNA Sequencing and Oligonucleotide Synthesis IBB, PAN, Warsaw, Poland) were used. The PCR reactions were carried in a 10 µl, containing 30 ng of genomic DNA template, 5 µl of 2x PCR Master Mix Plus (containing 0.1 U/µl Taq DNA polymerase, 4 mM $MgCl_2$ and 0.5 mM of each dNTPs; A&A Biotechnology, Gdansk, Poland), 1 µl of 10 µM primer and sterile, deionized water. The reaction were performed ATC401 Thermal Cycler (Nyx Technik Apollo, San Francisco, CA, USA) in the following reaction parameters: 92 °C for 5 min, followed by 35 cycles of denaturation at 92 °C for 1 min, primer annealing at 50.0–55.0 °C for 1 min., primer elongation at 72 °C for 2 min. The final extension step was 7 min at 72 °C. All the reactions were performed

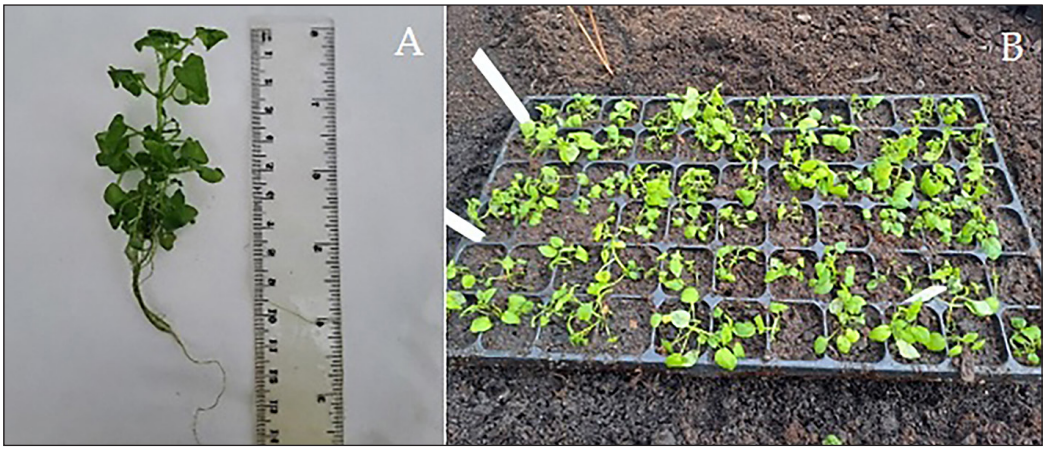

Figure 2. Microcuttings of *S. lateriflora* from *in vitro* culture conditions (A), acclimatization of microcuttings in greenhouse (B) (own study)

twice and the obtained products were separated in 1.6% agarose gel stained with ethidium bromide The analysis of the size of the obtained products was performed using a DNA ladder of 3000 bp (Genoplast Biochemicals, Rokocin, Poland) and the computer program GelAnalyzer 2010a (gelanalyzer.com). Gel Doc 2000 UV transilluminator (Bio-Rad, Hercules, CA, USA) was used to visualize and archive electrophoreograms.

RESULTS AND DISCUSSION

Shoot propagation

The micropropagation technique in *in vitro* culture allows for the rapid production of high-quality, disease-free and uniform planting materia (Kulus and Woźny, 2020; Magyar-Tábori et al., 2021; Baby et al., 2024). To initiate *in vitro* culture, explants taken from younger parts of the donor plant (shoots, cotyledons and leaves) are used because they have higher regenerative abilities compared to older tissue fragments. Seeds can also be used to establish an *in vitro* culture (Thiem and Kikowska, 2008; Szot et al., 2015). In the experiment carried out, seeds that had previously been chemically sterilized were used to establishment the *in vitro* culture of American skullcap, and after obtaining seedlings, single-node fragments of shoots were isolated from them and placed on MS medium. According to Koziara (2002), explants with good regenerative abilities can be isolated from young seedlings.

In the second passage, the developed axillary shoots were divided into single-node sections and inoculated onto MS medium with different combinations and concentrations of BAP and NAA. The greatest number of axillary shoots per 1 explant were obtained on MS medium containing 2 $mg \cdot dm^{-3}$ BAP (9.20). In this variant the multiplication factor (the number of axillary shoots obtained in relation to the number of rooted plants) was 15.3 (Table 1, Figure 3). The longest shoots with a length of 1.96 cm were recorded on MS medium which has been enriched of 1 $mg \cdot dm^{-3}$ BAP+0.5 $mg \cdot dm^{-3}$ NAA, and the shortest when 2 $mg \cdot dm^{-3}$ BAP was added to the medium. The obtained results are confirmed in the literature. According to Baby et al. (2024), enriching the medium with a high content of PGRs from the cytokinin group and at the same time a lower content of auxins affects the efficiency of plant multiplication in *in vitro* cultures. Cytokinins, an example of which is BAP used in the experiment, break the apical dominance and stimulate the development of axillary shoots.

The ability to develop side shoots from single-node explants of plants belonging to the genus *Scutellaria* in *in vitro* has been described by many scientists (Sinha et al., 1999; Zakaria et al., 2020; Dyduch-Siemińska and Gawroński, 2024). Sinha et al. (1999), examining the effect of BAP and NAA on the ability to develop shoots, showed that the mutual proportions of antagonistic phytohormones (cytokinin and auxin) regulate both the development of axillary shoots and affect the rhizogenesis of *S. discolor* plants. Dyduch-Siemińska and Gawroński (2024) obtained the highest multiplication factor (5.8) of *S. baicalensis* shoots in *in vitro* cultures in the variant in which the MS medium was enriched with BAP

Table 1. Effect of BAP and NAA on the formation of axillary shoots and callus tissue of American skullcap (*S. lateriflora*) in *in vitro* culture (passage II) (own study)

Variant	Plant growth regulator [mg·dm^{-3}]		Number of explants transferred to the growth medium	Average number of axillary shoots/ explant [pcs]	Mean number of explants forming shoots [%]	Mean axillary shoot length [cm]	Mean number of explants forming callus tissue [%]
	BAP	NAA					
A	0.0	0.0	13	1.91 ± 0.51	100	1.89 ± 0.41	0
B	1.0	0.0	11	6.55 ± 2.53	100	1.06 ± 0.38	82
C	2.0	0.0	10	9.20 ± 4.44	100	0.50 ± 0.00	100
D	1.0	0.5	13	3.82 ± 0.93	100	1.96 ± 0.49	100
E	2.0	1.0	8	2.50 ± 1.73	75	1.09 ± 0.78	100
	$LSD_{0.05}$			1.664	-	0.516	-

Note: results are mean ± SD (standard deviation); LSD – the lowest significant difference (Tukey's confidence half-interval) at $p \leq 0.05$; BAP – 6-benzylaminopurine, NAA – 1-naphthaleneacetic acid.

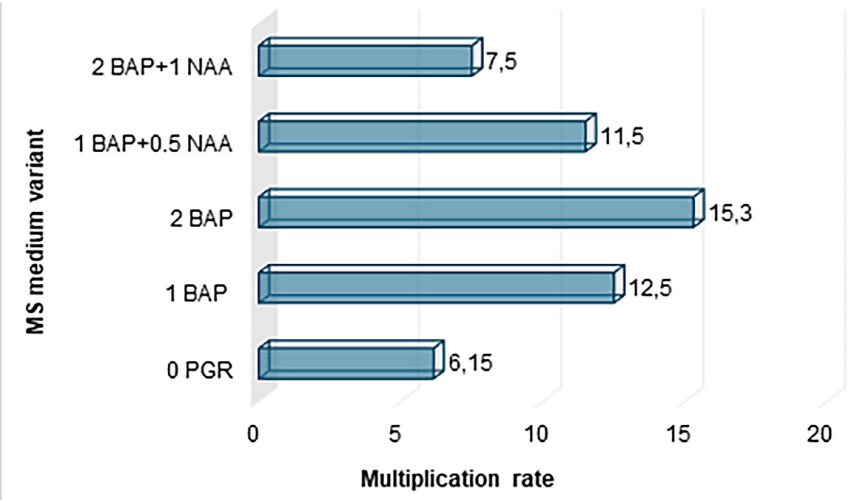

Figure 3. Multiplication rate of American skullcap (*S. lateriflora*) shoots in in vitro cultures (passage II): MS – Murashige and Skoog medium (1962), BAP – 6-benzylaminopurine, NAA – 1-naphthaleneacetic acid, PGRs – plant growth regulators.

in the amount of 1 mg·dm^{-3}. In turn, Zakaria et al. (2020) recorded the most axillary shoots from nodal explants in the MS medium variant enriched with 10.0 µM BAP + 0.1 µM NAA in the case of *S. alpina* and *S. altissima*. The authors compared cytokinins such as: BAP, MT (meta-topolin), KIN (kinetin), Zea (zeatin) and auxins: 2,4-D (2,4-dichlorophenoxyacetic acid) and NAA, in various combinations and concentrations.

Rooting and acclimatization

American skullcap shoots obtained from various variants of media were transferred to the rooting medium. For rooting, the basic MS medium without PGRs was used, which was the control in the experiment, and MS with IAA (indolyl-3-acetic acid) in the concentration of 0.5 mg·dm^{-3} (Figure 4). During of 21 days of observations, rhizogenesis was induced on all planted explants, both in the control variant and in the variant in which the substrate was enriched with PGRs. The efficiency of the rooting method in both experimental variants was 100% in both cases. Based on statistical calculations, more roots were recorded for the plant on the MS medium enriched with IAA compared to the control (Table 2). Research by other authors indicates that it is not necessary to enrich the MS medium with growth regulators for root formation. However, the addition of auxins at the rooting stage (IAA, IBA or NAA) significantly enhances this process (Tomaszewska-Sowa and Figas, 2011). Research by other authors confirms that the use of

Figure 4. Rooted plants of American skullcap (*S. lateriflora*) after 4 weeks of culture time

Table 2. Morphological characteristics of American skullcap (*S. lateriflora*) plants during rhizogenesis in vitro

MS medium	Number of explants [pcs]	Number of explants forming roots [%]	Average shoot length [cm]	Average root bundles length [cm]	Average number of roots [pcs]
0 PGR (control)	45	45 (100)	3.68 ± 1.15	7.86 ± 1.90	10.58 ± 4.93
0.5 mg·dm^{-3} IAA	45	45 (100)	3.18 ± 1.30	7.79 ± 2.32	13.62 ± 4.30
$LSD_{0.05}$	-	n.s.	n.s.	n.s.	2.030

Note: Results are mean ± SD (standard deviation); LSD – the lowest significant difference (Tukey's confidence half-interval) at $p \leq 0.05$; n.s – not significant; IAA – indole-3-acetic acid; MS – Murashige and Skoog medium (1962); PGRs– plant growth regulators.

IAA for rooting produced the expected results in the form of rooted plants with a larger number of roots compared to the control. Anbazhagan et al. (2010) compared the effectiveness of IAA, IBA, NAA on rooting of *Stevia rebaudiana*. IAA at a concentration of 1 mg dm^{-3} was found to be the most effective. According to Figas et al. (2025) for the species of *Leonurus sibiricus* plants, the addition of 0.5 mg·dm^{-3} of IAA also resulted in a higher number of roots compared to the control. In turn, Krupa-Malkiewicz and Mglosiek (2016) compared effect auxin (IAA, IBA and NAA) on

Figure 5. Plants of *S. lateriflora* in the greenhouse after about 2 weeks of acclimatization: growing in a multiplate containing horticultural peat (A); transferred to pots (B) (own study)

the rooting of *Celosia argentea* var. *cristata* (L.) and showed that IAA was less efficient compared to other auxins, however, when using 0.5 $mg \cdot dm^{-3}$ IAA, the authors observed longer roots.

Microcuttings of American skullcap were planted into a multiplate containing horticultural peat. A total of 90 plants were planted. After 14 days of growth, the acclimatized plants were planted individually into pots containing peat and left in greenhouse conditions for further growth. All plants adapted to greenhouse conditions (Figure 5a, Figure 5b, Figure 6).

Molecular assays

Analysis of the genetic homogeneity of plant materials obtained from *in vitro* cultures is a necessary aspect of the regeneration process (Vitamvas et al., 2019; Chávez-Cortazar et al., 2020). Genetic diversity in the genus *Scutellaria* was confirmed with use of PCR-based molecular markers, i.e., Inter Simple Sequence Repeats (ISSR) (Bai et al., 2013; Muraseva and Guseva, 2021), Random amplified polymorphic DNA (RAPD) (Su et al., 2008; Hosokawa et al., 2000; Shao et al., 2006), SSR (simple sequence repeat) markers (Yuan et al., 2015). Molecular analyses using the start codon targeting (SCoT) system were also performed to evaluate *Scutellaria baicalensis* regenerants obtained by indirect organogenesis (Gawroński and Dyduch-Siemińska, 2022) proves the mean similarity of the analyzed genotypes at the level of 0.90. The study demonstrated that the application of the SCoT markers allows to identify genetic variability in *S. baicalensis*, generated in *in vitro* cultures.

Figure 6. Plants of *S. lateriflora* in pots in a greenhouse, obtained by micropropagation after about 5 weeks of acclimatization (own study)

In the presented experiment to assess the genetic stability of S. *lateriflora* plants, a 15-point SCoT marker system was used *S. lateriflora* obtained through direct organogenesis of single-node shoot explants. 127 monomorphic products, which size ranged from 178 bp (SCoT-1) to 1083 bp (SCoT-9) were obtained as the result of the performed analyses (Table 3). The number of products generated by the starters from five (SCoT-27) to twelve (SCoT-1). The genetic homogeneity of the analyzed plants of American skullcap was demonstrated in all reactions regardless of the medium variants used in the experiment (Figure 7).

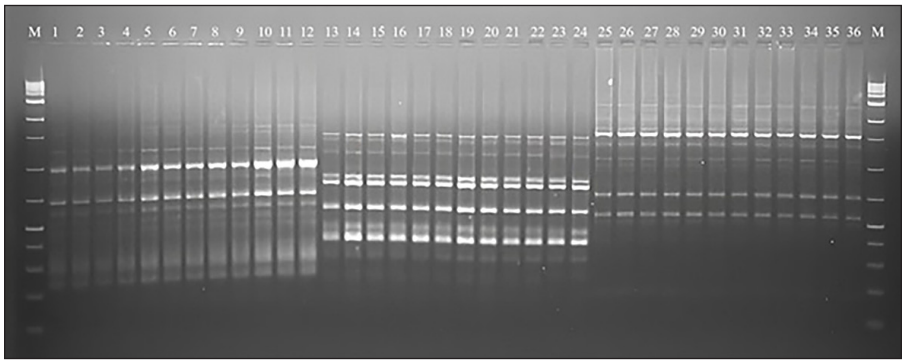

Figure 7. Selected SCoT band profiles of *S. lateriflora* accessions obtained in the experimental variants: A (lines: 1–2, 13–14, 25–26), B (lines: 3–4, 15–16, 27–28), C (lines: 5–6, 17–18, 29–30), D (lines: 7–8, 19–20, 31–32), E (lines: 9–10, 21–22, 33–34) and a seed–derived plants (lines: 11–12, 23–24, 35–36) generated by primers: SCoT-34 (1–12), SCoT-57 (13–24), SCoT-21 (25–36); M-3000 bp lader (own study)

Table 3. SCoT primers - used in molecular analysis of *S. lateriflora* accessions (own study)

Start codon targeted primer code	SCoT primers sequence [5'-3']	Annealing temperature [˚C]	No. of total products	Range of product size
SCoT-1	CAACAATGGCTACCACCA	53.0	12	178–793
SCoT-9	CAACAATGGCTACCACGT	50.0	10	421–1083
SCoT-11	AAGCAATGGCTACCACCA	50.0	9	359–784
SCoT-18	ACCATGGCTACCACCGCC	53.0	8	310–753
SCoT-21	CACCATGGCTACCACCAT	50.0	11	189–840
SCoT-23	CACCATGGCTACCACCAG	53.0	10	234–904
SCoT-25	ACCATGGCTACCACCGGG	53.0	9	347–821
SCoT-27	ACCATGGCTACCACCGTG	55.0	5	227–1040
SCoT-31	CCATGGCTACCACCGCCT	55.0	6	480–737
SCoT-34	ACCATGGCTACCACCGCA	53.0	8	234–809
SCoT-36	GCAACAATGGCTACCACC	50.0	7	522–824
SCoT-48	ACAATGGTACCACCAAGC	53.0	8	181–684
SCoT-57	ACAATGGCTACCACTACG	50.0	9	194–794
SCoT-70	ACCATGGCTACCAGCGCG	55.0	6	221–805
SCoT-79	CCATGGCTACCACTAGCT	55.0	9	347–827
Total	15 SCoT primers	50.0–55.0	127	178–1083 bp

Note: SCoT – Start Codon Targeted.

CONCLUSIONS

Micropropagation can be helpful in introducing new plant species because it allows for rapid and controlled propagation, which is especially useful when introducing rare or endangered species. In the conducted study, American skullcap (*S. lateriflora*) plants were obtained by direct organogenesis from explants of single-node shoot fragments obtained from sterile plants of this species. The highest efficiency of axillary shoot induction from explants isolated from sterile seedlings *S. lateriflora* recorded in the presence of 2 mg·dm^{-3} BAP in the MS medium (9.2). In this variant the multiplication factor (the number of axillary shoots obtained in relation to the number of rooted plants) was 15.3. Induction of rhizogenesis was achieved on all planted explants, both in the control variant (0 PGRs) and in the variant in which the medium was enriched with IAA in the amount of 0.5 mg·dm^{-3}. In the presented experiment, the SCoT molecular marker showed a high degree of genetic stability of micropropagated plants. Considering the large number of axillary shoots obtained from axillary buds located in the leaf axils of single-node shoot sections, as well as the efficient rooting process and the rapid acclimatization of the plants, this protocol can be used for mass multiplication of *S. lateriflora* plants.

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