Assiut Journal of Agricultural Sciences 55 (3) 2024 (78-96)
ISSN: 1110-0486 / EISSN: 2356-9840
Website: http://ajas.journals.ekb.eg/
E-mail: ajas@aun.edu.eg

(Original Article)

Assessment of Somaclonal Variants of *Salvia splendens* at Different Subcultures Using Molecular Markers

Mona A. Taha[1]; Azza A. Tawfik[1]*; Adel M. Mahmoud[2] and Mohamed F. Mohamed[3]

[1]Floriculture Department, Faculty of Agriculture, Assiut University, Assiut, Egypt.
[2]Agronomy Department, Faculty of Agriculture, Assiut University, Assiut, Egypt.
[3]Department of Vegetables, Faculty of Agriculture, Assiut University, Egypt.

*Corresponding author e-mail: azza7799@yahoo.com
DOI: 10.21608/AJAS.2024.279071.1349

Abstract

Genetic variability induced by cell and tissue culture techniques provides a new source of diversity for improvement of ornamental plants species. For this purpose, the effect of Thidiazuron (TDZ) on callus induction and number of subcultures of *Salvia splendens* was studied. The results showed that the MS medium supplemented with TDZ (0.5 mg/l) and Indole Acetic Acid IAA (0.1 mg/l) produced the highest percentage for explants producing callus (60%). For shoot multiplication, the best results were achieved when shoot tips were cultured on MS medium supplemented with TDZ (0.2 mg/l) alone. For subculture the same concentration of TDZ (0.2 mg/l) in media of eleven subcultures were performed at 21-day intervals over a total period of 231 days. For molecular variation detection, SCoT molecular markers were employed. Five SCoT primers were used and a total of 24 bands were obtained. The results showed high level of polymorphism between parent and their regenerated plants (subculture No. 11). These markers revealed distinctly different patterns of DNA fragments in all somaclones, and some distinctive bands were linked to the regenerated plants. For rooting, the best rooting percentage (100.00%), highest number of roots/shoot (7.00) and root length (2.58 cm) were observed when the shoots were cultured on MS hormone-free medium supplemented with 15 g sucrose/l. Plantlets were successfully acclimatized on Peat moss + perlite (1:1) with a survival rate of 100 %.

Keywords: *Micropropagation، Subcultures cycle، SCoT markers، Thidiazuron (TDZ).*

Introduction

The genus Salvia (Lamiaceae) consists of about 900 species including shrubs, herbaceous perennials, and annuals (Karousou *et al*., 2000).

Salvia splendens is commonly known as Scarlet sage (Shanker and Unnikrishnan 2001; Bobbi 2003) and considered as one of the most commonly observed ornamental perennial plants in the landscape (Erv, 2000). It is characterized by a wide color variation, long-lasting flowering, heat tolerance and resistance to pests and diseases (Regnault, 1997; Alberto *et al.,* 2003). *Salvia splendens* is a worldwide popular bedding plant with a significant economic value

Received: 28 Mars 2024/ Accepted: 21 April 2024/ Published online: 21 July 2024

and widely cultivated in many countries around the world (Stepankova, 2001). *Salvia splendens* is among the most commonly cultivated herbaceous ornamental plants and frequently planted for edging walkways and borders in gardens, flowerbeds, and by roadsides (Soundararajan *et al.,* 2013). Therefore, it is of great practical importance to breed new varieties to meet the demand of market for new high-quality varieties (Hui *et al.,* 2004; Fu *et al.,* 2009; and Chen *et al.,* 2016).

As a seasonal flower with decorative values and such worldwide importance in landscaping, micropropagation and tissue culture techniques can provide the plant breeders protocols and methods for plant genetic manipulation of *Salvia splendens*. During in vitro culture, many factors may affect the fidelity of the genotypes leading to the occurrence of somaclonal variation i.e., genetic and epigenetic variation (Larkin and Scowcroft 1981; Cassells, 2002; Bairu *et al.,* 2011; Ferreira *et al*., 2023). These factors include: plant genotypes, the explant source, the type and concentration of plant growth regulators, propagation methods and the number as well as the interval of subcultures (culture age). Such, variations could demonstrate improvement in the quality of genotype and its yield.

Several methods (morphological, cytological, biochemical and molecular methods) have been reported to detect somaclonal variants (Bairu *et al.,* 2011; Ranghoo-Sanmukhiya, 2021). A number of PCR based molecular markers like random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), simple sequence repeat (SSR) and start codon targeted (SCoT) polymorphism are preferred for the detection of somaclonal variation (Rai, 2022). Because of its many advantageous characteristics, and its many desirable features the start codon targeted (SCoT) polymorphism marker has gained significant importance in plant genetics, genomics, and molecular breeding. Targeted by the SCoT marker is the highly conserved area of plant genes that surrounds the start codon Start codon targeted (SCoT) polymorphism marker. SCoT marker targets the region nearby the start codon, a highly conserved region in plant genes (Collard and Mackill 2009; Rai, 2023).

This study was conducted for assessment the morphological and molecular variation of in vitro-propagated plants of *Salvia splendens* at different subcultures using SCoT molecular markers.

Materials and Methods

This work was carried out at plant tissue culture laboratory, Department of Ornamental Plants and Landscape Gardening, Faculty of Agriculture, Assiut University.

Plant material and in vitro culture establishment

Shoot tips of *Salvia splendens was* collected from recent branches growing in greenhouse of the nursery of ornamental plants Faculty of Agriculture, Assiut University. The healthy shoot tips (0.5cm) were washed thoroughly in running tap water for 30 min. Then, they were transferred to laminar flow hood for sterilization using 30% commercial bleach (5% NaOCl) for 20 minutes. The ends of the shoot

tips were cut and all the explants (0.3- 0.5 cm) were rinsed three times using sterile distilled water and cultured in 250 ml jars containing 30 ml of MS medium (Murashige and Skoog, 1962).The MS medium was supplemented with 3% (w/v) sucrose and 8 g/l agar and the pH of the medium was adjusted to ± 5.8 before autoclaving at 121º C and 1.5 kg/cm^2 for 20 min. The cultures were incubated at 24 ± 2°C for 16 hours under light of cool white fluorescent tubes.

Callus induction

Shoot tips (~ 0.3 – 0.5 cm) were used as explants and grown on MS basal medium supplemented with Thidiazuron (TDZ) at different concentrations (0.0, 0.25, 0.5 and 1 mg/l) combined with Indole Acetic Acid (IAA) at 0.00, 0.10 mg/l. The percentage of explant producing callus, its color and texture were observed and recorded after 4 weeks. The factorial experiment (2 × 4) was carried out in a randomized complete block design (RCBD) with three replicates per treatment and five jars per replicate each jar containing 2 explants.

Adventitious shoot from callus explants

Pieces (~0.5 g) of callus were cultured onto MS medium supplemented with 30 g/l sucrose and different concentrations of TDZ (0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/l). Data were obtained after 4 weeks on percentage of regeneration, Shoots number/ explant and leaf number/ explants of *Salvia splendens.*

An experiment in a completely randomized design (CRD) with three replicates was used. Each replicate comprised five jars, each jar containing two pieces of callus.

Shoot multiplication

Shoot tips (~1 – 1.5 cm) were excised from the regenerated shoots of callus. Two shoots were cultured onto a 250 ml baby-food jar filled with 30 ml MS basal medium supplemented with 30 g/l sucrose and different concentrations of TDZ (0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/l). After 4 weeks of culture, data on regeneration percentage, number of shoots/ explant, shoot length (cm), and number of leaves/shoot were recorded. The plants resulting from this stage were used for next stage.

Subculture cycles during *Salvia splendens* micropropagation

To assess how is the number of subculture cycles affect somaclonal variation? The multiplication rate (expressed as a number of shoots/explant) during micropropagation of *Salvia splendens* shoots were transfer from multiplication stage and subculture on fresh MS medium supplemented with 0.2 mg/l (TDZ). Two shoots were placed in each vessel and five vessels were used per replicate. Regeneration percentage, number of shoots/ explants and shoot length were registered. Over a period of 231 days, eleven subcultures were carried out at intervals of 21 days. The experiment was arranged in a complete randomized design (CRD) with three replicates.

Experimental design and data analysis

For all the experiment, data were subjected to analysis of variance (ANOVA), and mean comparisons were performed using the LSD at probability level of 5% (Gomez and Gomez, 1984).

Detecting of somaclonal variation and molecular assays

The plant samples (0.5 g/ each) were excised from the leaves of mother plant and leaves from shoots grown in each subculture (No.2 to subculture No.11) were used for DNA extraction.

DNA extraction

Total genomic DNA of the studied samples, was extracted from fresh leaves, using Cetyl trimethyl ammonium bromide (CTAB) protocol for plants (Murray and Thompson, 1980). Concentration and quality of DNA was measured at 260/280 nm using a spectrophotometer and checked by separating DNA on 0.8% agarose gel. Each sample was diluted to 50 ng/µl with TE buffer (10mM Tris–HCl, pH 8.0 and 0.1mM EDTA, pH 8.0) and stored at 4 ◦C for further use.

SCoT markers analysis

Five start codon targeted (SCoT) primers (Table 4), (metabion international AG) were used to amplify the DNA template of our samples. The amplification PCR reactions were carried out in 25µl final volumes, containing (11.0 µl dH_2O, 3 µl of 10x buffer, 3.0 µl of dNTPs (2.5mM) 4 µl of Mg C12 (25 m M), 3.0 µl primer (2.5µl), 0.3 µl of Taq polymerase (5U/ µl) and 2.0 µl of genomic DNA (50 ng/ml). Amplification of SCoT primers was performed in a programmed thermocycler (C-1000) with initial denaturation at 94◦C for 3min, 36 cycles of denaturation at 94◦C for 1min, primer annealing at 51◦C for 1min, extension at 72◦C for 1min, and final extension at 72◦C for 3min.

Molecular data analysis

Five primers for SCoT provided distinct and robust band patterns. The DNA banding patterns generated by SCoT markers were analyzed and the presence (1) or absence (0) of each band was recorded for each sample for all the tested primers. Using NTsys PC software, a separate cluster analysis based on SCoT data was conducted, and a dendrogram was created according to Jaccard's (1908) coefficient for the unweighted pair-group method with arithmetic averages (UPGMA). Furthermore, data on a dendrogram displaying SCoT markers was also obtained. Moreover, some diversity parameters were calculated from molecular data of all markers, including percentage of polymorphism (%P), polymorphism information content (PIC), primer resolving power (RP), diversity index (DI) and marker index (MI) (Nei, 1987; Prevost and Wilkinson,1999; Roldan-Ruiz *et al.,* 2000; Varshney *et al.,* 2007).

Rooting stage

The micro-shoots (from each subculture) were tested to induce roots and obtain complete plantlets to be hardened and transferred to the field with a minimum loss. So, shoots of 3-4 cm in length were cultured on half-strength MS

medium without PGRs supplemented with 15 g/l sucrose. Rooting percent, number of roots/plant and root length (cm) were recorded.

Acclimatization stage

The rooted plantlets (~4 cm in length) were gently removed from the culture medium, then the roots were rinsed thoroughly with tap water to remove the excess medium. All the plantlets were transplanted into small plastic pots (10 cm diam.) filled with a mixture of peat moss and perlite (1:1 v/v). Four weeks later, data on the survival rate of the plants was calculated.

Results and Discussion

Effect of PGRs on culture establishment and callus induction

Data presented in Table 1 showed significant effects among concentrations of TDZ with IAA for callus formation of *Salvia splendens*. A green compact callus was induced from shoot tips of *Salvia splendens* cultured on media supplemented with any concentration of TDZ used or when IAA at 0.1 mg/l was combined with TDZ at all concentrations used. Meanwhile, the PGRs-free medium did not produce callus (Table 1). The medium supplemented with TDZ at 0.5 mg/l plus IAA at 0.1 mg/l resulted in the highest percentage for explants producing callus (60%). (Huii *et al.,* 2012) reported that the highest rate of callus induction from leaf and stem explants of *S. splenden*s occurred in the medium containing 1.0 mg/ L 2,4-D and 1.5 mg/l BAP. On the other hand, (Yu *et al.,* 2015) explained that the whole plantlets of *Salvia splendens* could be rapidly regenerated without intermediate callus stage to avoid the occurrence of any genetic or and epigenetic variation. Many investigations report protocols for direct regeneration by organogenesis and clonal propagation for commercial industry of Salvia published by many Authors (Sharma *et al.,* 2014; Kabir *et al.,* 2014; Yu *et al.,* 2015 and Papafotiou *et al.,* 2023).

Table 1. Effect of different concentrations of TDZ and IAA on callus induction of *Salvia splendens* after four weeks in culture

TDZ mg/l / IAA mg/l	Explants producing callus %					Visual observations on callus characteristics			
	0.00	**0.25**	**0.50**	**1.00**	**Mean**	**0.00**	**0.25**	**0.50**	**1.00**
0.0	0.0	10.0	30.0	40.0	20.0	----------	Friable and green	green	Friable and green
0.1	0.0	30.0	60.0	40.0	32.5	----------	Compact green callus	Compact green callus	Compact green callus
Mean	0.0	20.0	45.0	40.0					
LSD 0.05									
TDZ					7.94				
IAA					5.61				
TDZ x IAA					11.22				

TDZ: Thidiazuron, IAA: Indole Acetic Acid

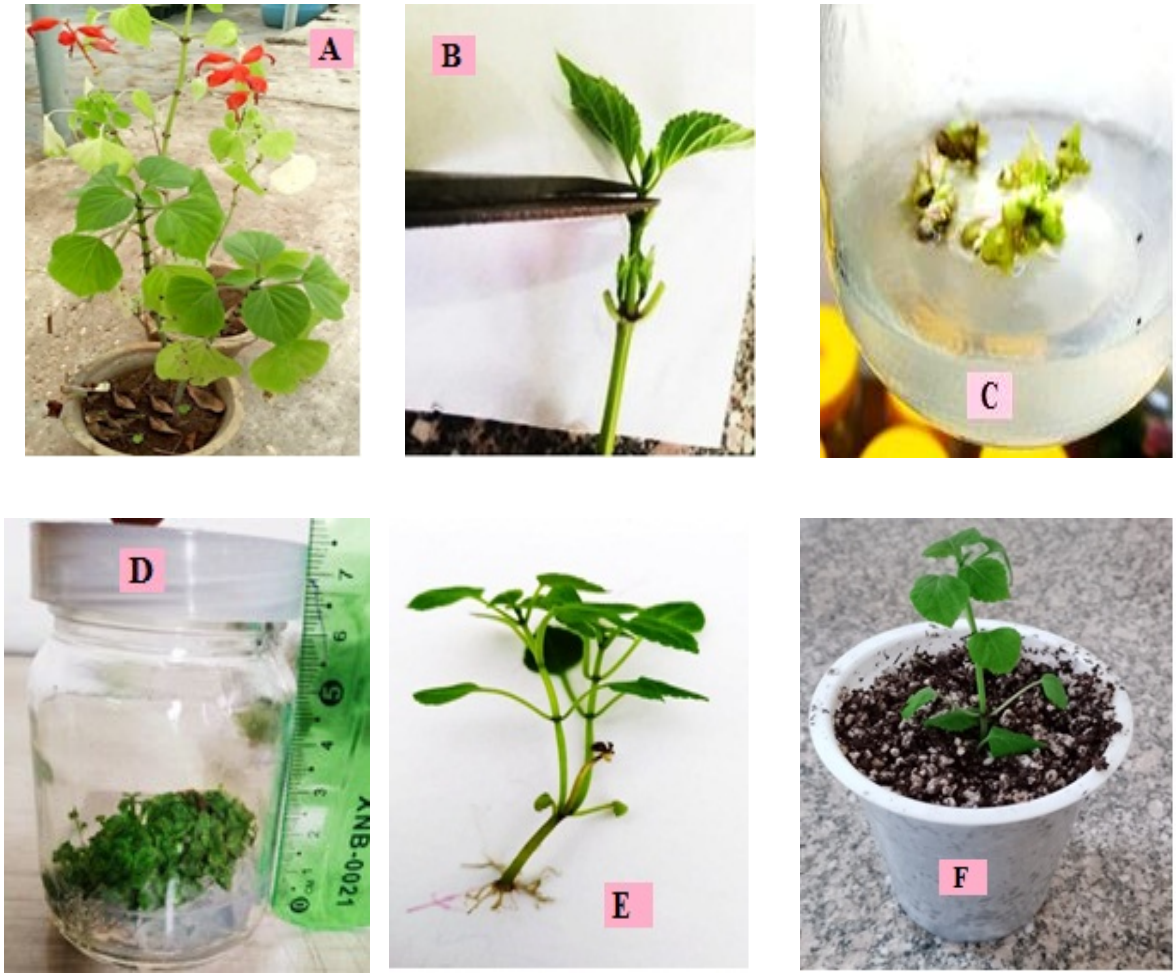

Figure 1. *In vitro-propagated Salvia splendens*. (A) Mother plant of Salvia splendens; (B) Explant (Shoot tips); (C) callus induction on MS medium with 0.25 mg/l TDZ with 0.1 IAA after 4 weeks; (D) Adventitious shoot multiplication on medium with 0.2 mg/l TDZ after 4 weeks (E). Rooting of isolated single shoot on 1/2 MS medium with 15 g sucrose/l; (F) Acclimatized plantlets transferred to plastic pots filled with Peat moss + perlite (1:1) after 3 weeks.

Effect of TDZ on adventitious shoot from callus explants

Data in Table 2 revealed significant effects on shoots regenerated form callus of *Salvia splendens* when callus was cultured on medium supplemented with different concentration of TDZ (0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/l). The regeneration percentage was ranged from 10% (control treatment without TDZ) to 100% (TDZ at 0.4 mg/l). However, Applying TDZ from 0.2 up to 0.5 mg/l produced the highest regeneration rate with no significance difference among them. TDZ (0.2 mg/l) produced the highest number of shoots (16 shoots/explant), shoot length (3.61cm) and leaves-number (28 leaves /shoots) with regeneration rate of 90%.

The regenerated shoots look normal and no phenotypic change was observed at this stage. Indirect regeneration methods through the callus induction could

result in morphological genetic and epigenetic variations (Maruthi *et al.,* 2012; and Huii *et al.,* 2012). De Souza *et al.,* 2021 reported that callus formation during micropropagation may have been responsible for the abnormality in 5% of the regenerated plants.

Table 2. Effect of different concentrations of Thidiazuron (TDZ) on shoots regenerated from callus explants of *Salvia splendens* after four weeks in culture.

TDZ mg/l	Regeneration %	Shoot number/ explant	Shoot length (cm)	Leaves number/ explant
0.00	10.00	1.00	2.25	3.00
0.10	70.00	4.20	3.34	19.00
0.20	90.00	16.00	3.61	28.00
0.30	90.00	13.00	3.13	31.00
0.40	100.00	9.00	3.44	23.00
0.50	90.00	5.50.	4.11	17.00
L.S.D 5%	**12.58**	**1.13**	**0.52**	**4.04**

Effect of TDZ on shoot multiplication

The regenerated adventitious shoots excised from callus formed in the previous stage were multiplied on MS medium supplemented with different concentrations of TDZ (0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/l). It is clear from the results represented in Table 3, that using TDZ (0.2 mg/l) induced the best number of shoots/explant (18.00 shoots), shoot length (5.41 cm) and number of leaves/explant (44.00 leaves) with 100% regeneration. Sharma *et al.,* 2014 mentioned that the ability of *S. splendens* explants to form new shoots varied with the explant's type and cytokinin used. They observed that BA (5.0 µM) was more effective than other cytokinins (Kinetin, 2-iP and TDZ) in inducing shoot regeneration of *Salvia splendens*. However, they observed that shoot tips when cultured on MS medium with TDZ at 0.1 mg /l produced 3.8 shoots/explant shoot length (1.9 cm) and 82% regeneration. The application of plant growth regulators and endogenous plant hormons are factors that can induce genetic variability. The endogenous accumulation of growth regulators in the explant that favors an increase in the multiplication rate during the subculture cycles may cause inhibition of growth and variation, and subsequence the excess of growth regulators may act as a mutagenic agent (Ferreira *et al.,* 2023).

Sales and Butardo (2014), reported that, several growth regulators, such as NAA, 2,4-D and BAP may be responsible for obtaining in vitro genetic instability. Some studies have shown that TDZ are responsible for somaclonal variants (Bairu *et al.,* 2006; Bidabadi, *et al.,* 2010 and Samarfard *et al.,* 2014).

Table 3. Effect of different concentrations of Thidiazuron (TDZ) on multiplication of shoots of *Salvia splendens* after four weeks in culture

TDZ mg/l	Regeneration %	Shoot number/ explant	Shoot length (cm)	Leaves number/ explant
0.00	70.00	2.00	2.15	5.00
0.10	90.00	11.00	4.26	32.00
0.20	100.00	18.00	5.41	44.00
0.30	100.00	17.00	5.23	35.00
0.40	100.00	12.00	3.84	25.80
0.50	100.00	14.00	4.50	31.00
L.S.D 5%	**10.27**	**2.91**	**0.87**	**2.67**

Effect of subculture cycles on shoot multiplication

Eleven subcultures were performed at 21-day intervals over a total period of 231 days. The number and length of shoots showed significant differences among subculture cycles (Fig 2). The reduction in number of shoots per explant was observed from subcultures 9 to 11(Fig 2 b), while a significant decline in shoot length was observed in subculture 10 and 11 (Fig 2 c). At the phenotypic level, some genotypes remained stable over five subcultures (de Souza *et al.,* 2021). Regarding the regeneration rate, there were no significant differences among subculture cycles from 1 to 10 but at subculture 11 (Fig. 2 a), a significant reduction was observed (80 %) compared to subculture 1 (100%).

Morphological variations in banana plants subjected to micropropagation were observed at 10th subculture cycle (Khan *et al.,* 2011). They observed a reduction in the multiplication rate and an increase in somaclonal variation frequency after eight subcultures. The effect of subculture frequency on genetic variations has been investigated by several researchers (Peng *et al.,* 2015; Graner *et al.,* 2019; Pastelin-Solano *et al.,* 2019 and Hesami *et al.,* 2023). The results obtained in this study showed that subculture cycles have an effect on the regeneration rate, shoot length and number of shoots in *S. splendens* micropropagation. Similar results obtained by Hesami *et al.,* (2023) showed that regeneration rate and shoot length have been shown to decrease as the number of subcultures increases. This may be due to endogenous hormone content (Peng *et al.,* 2015).

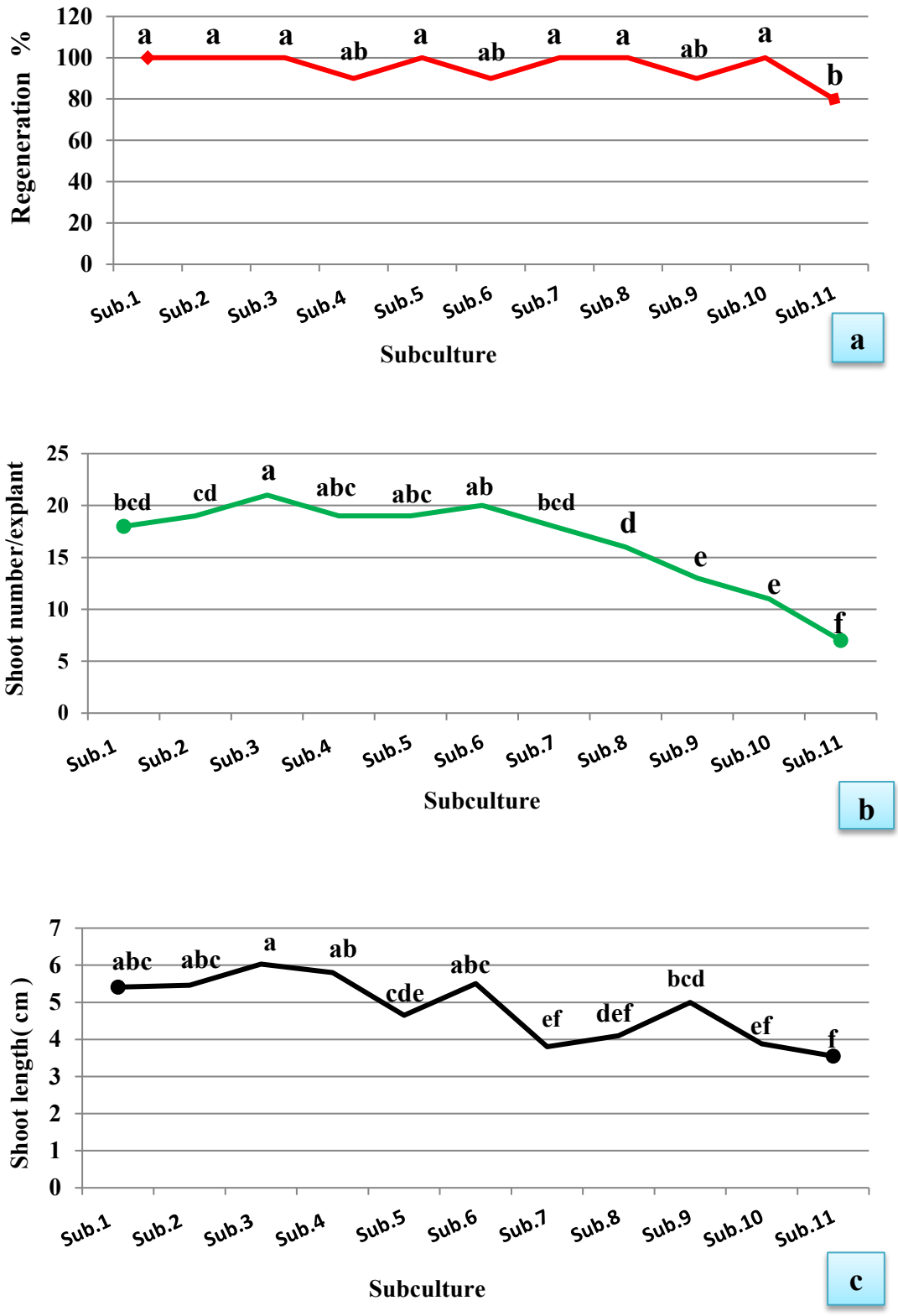

Figure 2. Effect of subculture cycles on shoot regeneration (a), shoot number/explant (b) and shoot length cm (c) during micropropagation of *Salvia splendens* cultured in MS + 0.2 mg/l TDZ. a,b..f: different letters means significant differences at 0.05

Effect of subculture cycles on somaclonal variation and molecular assays

Five SCoT (Table 4) provided repeatable outcomes and polymorphic bands, which were taken into consideration for data analysis. Distinct differences in the reproducible bands obtained from samples (from the mother plant and from subculture No.2, subculture No.11) are shown in Figure 3 and Table 4.

Table 4. Primer sequences and codes used in this study

Primer codes		Sequence (5' to 3')	Temp. (ºC)
SCoT	SCoT-10	CAACAATGGCTACCAGCC	53
	SCoT-18	ACCATGGCTACCACCGCC	54
	SCoT-22	AACCATGGCTACCACCAC	53
	SCoT-28	CCATGGCTACCACCGCCA	54
	SCoT-32	CCATGGCTACCACCGCAC	53

Furthermore, a number of diversity metrics were computed using the marker data, including polymorphism information content (PIC), primer resolving power (RP), diversity index (DI), and marker index (Table 5).

Using several plant entries, SCoT amplified a total of 21 DNA fragments. The molecular size range of the SCoT-PCR products was from 720 bp (SCoT-10) to 1580 bp (SCoT-32) with an average of 4.2 fragments per primer **(**Figure 3). Out of the 21 fragments scored, 10 bands (48.00%) were found to be polymorphic and 11 loci (52.00 %) were found to be monomorphic in nature with an average of 2.00 polymorphic bands per primer (Table 5).

The polymorphism percentage (% P) ranged between 25.00% (for SCoT-10) and 100% (for SCoT-22). The polymorphic information content (PIC) values for the five SCoT primers varied from 0.11 (SCoT-10) to 0.44 (SCoT-22). The marker index (MI) values ranged from 0.11 (SCoT-10) to 2.20 (SCoT-22). The resolving power of primer (RP) values ranged from 0.66 to 3.30, the SCoT-22 primer gave the highest RP values (3.30) while SCoT-18 exhibited the lowest RP value (0.66) (Table 5). The diversity index (DI) values ranged from 0.37 (SCoT-18) to 1.75 (SCoT-22). These markers revealed distinctly different patterns of DNA fragments in all somaclones and some distinctive bands were linked to the regenerated plants in all of the molecular markers examined. This may be due to somaclonal variations which were notable in their differences from the parents Rai, (2022).

Table 5. Genetic variation parameters produced with SCoT DNA markers [(1)]

Marker	Primer	TB	NPB	%P	PIC	RP	DI	MI
SCoT	**SCoT-10**	4	1	25.00	0.11	0.66	0.37	0.11
	SCoT-18	2	0	00.00	0.00	0.00	0.00	0.00
	SCoT-22	5	5	100.00	0.44	3.30	1.75	2.2
	SCoT-28	3	2	67.00	0.29	1.32	0.75	0.58
	SCoT-32	5	2	40.00	0.18	1.32	0.64	0.36
	Total	21	10	--	--	--	--	--
	average	4.2	2.00	46.40	0.204	1.32	0.70	0.65

[(1)] TB= Total Bands, NPB= Number of Polymorphic Bands, %P= percentage of polymorphism, PIC= polymorphic information content, RP= ResolvVing Power, DI= diversity index, MI= Marker Index.

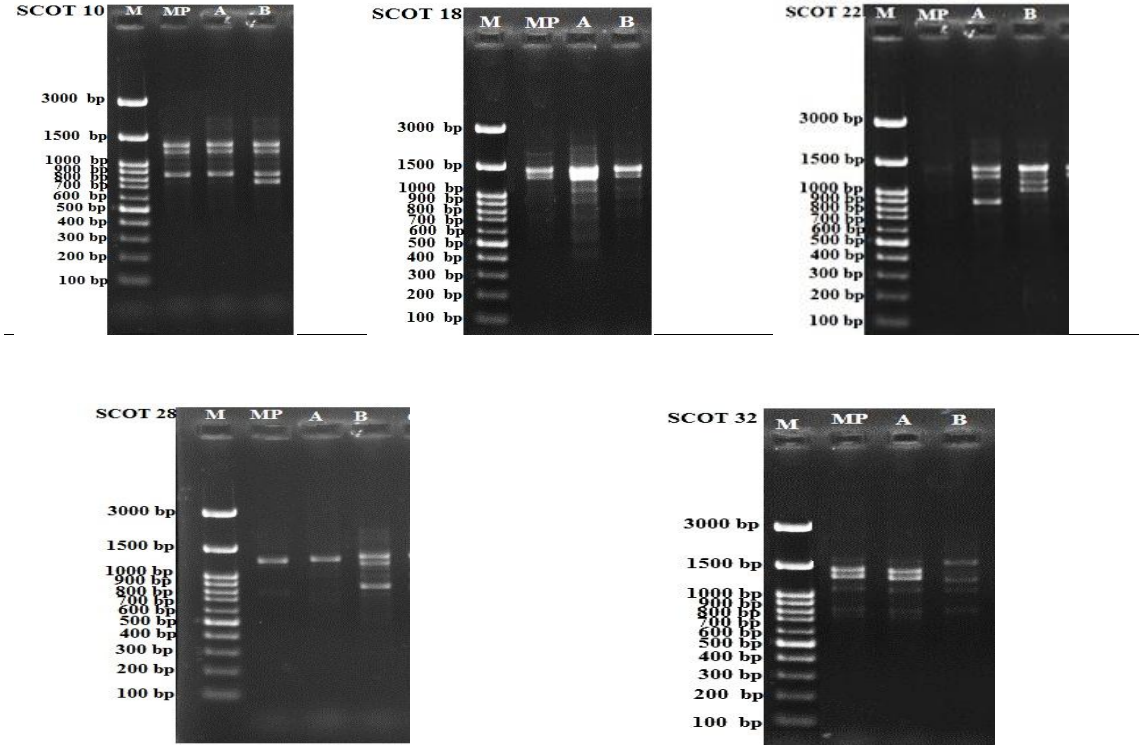

Figure 3. SCoT banding profile of *Salvia splendens*. M: represented the DNA ladder, MP: Mother plant, A: proliferated plants from subculture-2, B: proliferated plants from subculture-11

The three samples were divided into two main groups by the dendrogram (Figure 4). Mother plant (MP) and proliferated plants from subculture-2 (A) with similarity (0.769) are found in group 1. Proliferated plants from subculture-11 (B) with node 1 (MP+ A) and cluster-2 included with a similarity of 0.528.

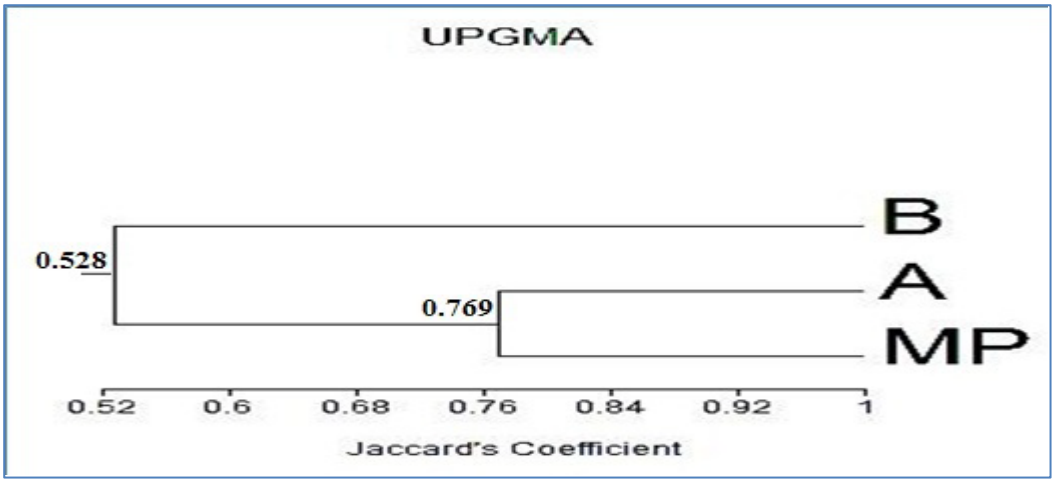

Figure 4. A dendrogram was created by applying UPGMA analysis to the data from five SCoT primers. The scale depends on the coefficients of Jaccard. MP: Mother plant, A: proliferated plants from subculture-2, B: proliferated plants from subculture-11

Molecular analysis has detected somaclonal variation in many species (Pastelín Solano *et al*., 2019; Tikendra *et al*., 2021; Gawroński and Dyduch-Siemińska 2022; and Rai 2022). Khan *et al*., (2011), in Musa spp., reported that after subculture eight, the percentage of somaclonal variants was detected using SSR markers. Farahani *et al*., (2011), in *Olea europea*, observed morphological change and genetic variation among the regenerated plants after subculture seven. Similar results were reported in Stevia rebaudiana by Soliman *et al*., (2014) and *Pistacia lentiscus* by Kılınc¸ *et al*., (2015), in which the greatest genetic instability was detected in the highest number of subcultures. Somaclonal variation has been detected in many micropropagated plant species using ISSR markers (Bello-Bello *et al.,* 2014; Martı´nez-Estrada *et al.,* 2017; Babu *et al.,* 2018). However, SCoT markers are gaining popularity over other dominant DNA marker systems like RAPD and ISSR for higher polymorphism and better marker resolvability (Gorji *et al.,* 2011; Satya *et al.,* 2015; Shalan *et al.,* 2023). Moreover, Antony *et al.,* (2015) used start codon targeted polymorphism (SCoT) DNA markers for the identification of somaclonal variation in cryopreserved *Dendrobium* Bobby Messina.

Rooting and Acclimatization of regenerated shoots

As shown in Figure 1 E shoots were easily rooted (100%) after 3 weeks when cultured on half-strength MS medium supplemented with sucrose at 15 g/l. Acclimatization of plantlets is a vital step in the micropropagation protocol of any plant species. It involves the transfer of plantlets from tissue culture containers to *ex vitro* conditions. In the present study, a plantlet of *Salvia splendens* was acclimatized as shown in Figure 1 F. The plantlets were transferred to plastic pots with peat moss and perlite (1:1 v/v) after four weeks. Plantlets were successfully acclimatized with 100 % survival rate.

Conclusion

In conclusion, the subculture number affects somaclonal variation and in vitro development of *Salvia splendens*. In our study, the polymorphism found after the eleven subculture in MS medium supplemented with 0.2 mg/l TDZ suggests that it could be a promising protocol for plant breeders. However, if the objective is to maintain high genetic fidelity during micropropagation for commercial purpose, it is recommended that in shoot multiplication stage no more than five subcultures. For further studies of micropropagation of *Salvia splendens* on a commercial scale, it is recommended that number of subculture must be mentioned.

References

Alberto, C. M.; Sanso, A.M. and Xifreda, C. C. (2003). Chromosomal studies in species of Salvia (Lamiaceae) from Argentina. Botanical Journal of the Linnean Society, 141(4): 483-490.

Antony, J. J. J.; Shamshir, R. A.; Poobathy, R.; Chew, B. L. and Subramaniam, S. (2015). Somaclonal variations were not induced by the cryopreservation: Levels of somaclonal variations of in vitro and thawed protocorms of Dendrobium Bobby

Messina analysed by SCoT and TRAP DNA markers. South African journal of botany, 100: 148-157.

Babu, G.A.; Vinoth, A. and Ravindhran, R. (2018). Direct shoot regeneration and genetic fidelity analysis in finger millet using ISSR markers. Plant Cell, Tissue and Organ Culture (PCTOC), 132:157-164.

Bairu, M.W.; Aremu, A.O. and Van Staden, J. (2011). Somaclonal variation in plants: causes and detection methods. Plant Growth Regulation, 63: 147-173.

Bairu, M.W.; Fennell, C.W. and van Staden, J. (2006). The effect of plant growth regulators on somaclonal variation in Cavendish banana (*Musa AAA cv.'Zelig'*). Scientia Horticulturae, 108(4): 347-351.

Bello-Bello, J.J.; Iglesias-Andreu, L.G.; Avilés-Vinas, S.A.; Gómez-Uc, E., Canto-Flick, A. and Santana-Buzzy, N. (2014). Somaclonal variation in habanero pepper (*Capsicum chinense Jacq*.) as assessed ISSR molecular markers. HortScience, 49(4): 481-485.

Bhojwani, S.S. and Dantu, P.K. (2013). Somaclonal variation in plant tissue culture: an introductory text. Springer, New York, 141–152.

Bidabadi, S.S.; Meon, S.; Wahab, Z. and Mahmood, M. (2010). Study of genetic and phenotypic variability among somaclones induced by BAP and TDZ in micropropagated shoot tips of banana (*Musa spp*.) using RAPD markers. Journal of Agricultural Science, 2(3): 49.

Bobbi, A. (2003). Splendid salvias-14 Tender Perennials for summer and fall Bloom. Brooklyn Botanic Garden, New York, 18(3).

Cassells, A.C. (2002). Tissue culture for ornamental breeding. In Breeding for ornamentals: classical and molecular approaches. Dordrecht: Springer Netherlands. 139-153.

Chen, Y.i.; Chun-nan, L.I.; Qiao-juan, F.U.; Fu-kang, Z.H.A.O. and Yao, S.U.N. (2016). A new scarlet sage cultivar 'Hongyun'. Acta Horticulturae Sinica, 43(S2): 2789-2790.

Collard, B.C. and Mackill, D.J. (2009). Start codon targeted (SCoT) polymorphism: a simple, novel DNA marker technique for generating gene-targeted markers in plants. Plant molecular biology reporter, 27: 86-93.

De Souza, R.; Adams, C.R.; de Melo, R.C.; Guidolin, A.F.; Michel, A. and Coimbra, J. L. M. (2021). Phenotypical changes in hop induced by micropropagation. Plant Cell, Tissue and Organ Culture (PCTOC), 147(2): 379-387.

Devecchi, M. (2006). The use of Labiatae of ornamental interest in the design of parks and gardens. In I International Symposium on the Labiatae: Advances in Production, Biotechnology and Utilisation, 723: 51-58.

Dong, A.X.; Xin, H.B.; Li, Z.J.; Liu, H.; Sun, Y.Q.; Nie, S. and Mao, J.F. (2018). High-quality assembly of the reference genome for scarlet sage, Salvia splendens, an economically important ornamental plant. GigaScience, 7: 7.

Erv, E. (2000). *Salvia splendens*, Consumer horticulturist . NC State University, Florida, 2000- 2003(www.Salviasplendens.com).

Espinosa-Leal, C.A.; Puente-Garza, C.A. and García-Lara, S. (2018). In vitro plant tissue culture: means for production of biological active compounds. Planta, 248: 1-18.

Farahani, F.; Yari, R. and Masoud, S. (2011). Somaclonal variation in Dezful cultivar of olive (*Olea europaea subsp. europaea*). Gene Conserve, 10:216–221.

Ferreira, M.D.S.; Rocha, A.D.J.; Nascimento, F.D.S.; Oliveira, W.D.D.S.; Soares, J.M.D.S.; Rebouças, T.A. and Amorim, E.P. (2023). The role of somaclonal variation in plant genetic improvement: A systematic review. Agronomy, 13(3): 730.

Fu QiaoJuan, F.Q.; Shen GuoZheng, S.G.; Chen Yi, C.Y. and Zhao HangPing, Z.H. (2009). A new scarlet sage cultivar'Shenzhouhong'. Acta Hort. Sinica 36:1557–1558.

Gomez, K.A. and Gomez, A.A. (1984) "Statistical Procedures for Agricultural Research ", 2nd ed., Wiley, New York, 680 p.

González, A.I.; Saiz, A.; Acedo, A.; Ruiz, M.L. and Polanco, C. (2013). Analysis of genomic DNA methylation patterns in regenerated and control plants of rye (*Secale cereale L.*). Plant Growth Regulation, 70: 227-236.

Gorji, A.M.; Poczai, P.; Polgar, Z. and Taller, J. (2011). Efficiency of arbitrarily amplified dominant markers (SCoT, ISSR and RAPD) for diagnostic fingerprinting in tetraploid potato. American journal of potato research, 88: 226-237.

Graner, E.M.; Calderan-Meneghetti, E.; Leone, G.F.; de Almeida, C.V. and de Almeida, M. (2019). Long-term in vitro culture affects phenotypic plasticity of Neoregelia johannis plants. Plant Cell, Tissue and Organ Culture (PCTOC), 137: 511-524.

Hesami, M.; Adamek, K.; Pepe, M. and Jones, A.M.P. (2023). Effect of explant source on phenotypic changes of in vitro grown cannabis plantlets over multiple subcultures. Biology, 12(3): 443.

Hui ChangMin, H.C.; Han Xuan, H.X.; Liang ZhaoHui, L.Z.; Zheng ChunYu, Z.C.; Ma ChunYang, M.C. and Liu Libo, L.L. (2004). A new variety of scarlet sage-'Zhongchuanhong'. Acta Hort. Sinica 31:281.

Huii, L.; Guoping, Z.; Guozheng, S.; Songlin, R. and Qiaojuan, F. (2012). Callus Induction and Plant Regeneration from Mature Seeds of *Salvia splendens*. International Journal of Agriculture & Biology, 14(3) 445-449.

Jaccard, P. (1908). Nouvelles recherches sur la distribution florale. Bull. Soc. Vaud. Sci. Nat., 44: 223-270.

Jain Mohan, S. (2001). Tissue culture-derived variation in crop improvement. Euphytica, 118(2):153–166.

Jain, S.M.; Brar, D.S. and Ahloowalia, B.S. (Eds.). (2013). Somaclonal variation and induced mutations in crop improvement (Vol. 32). Springer Science & Business Media, Berlin,32.

Jia, K.H.; Liu, H.; Zhang, R.G.; Xu, J.; Zhou, S.S.; Jiao, S. Q. and Mao, J.F. (2021). Chromosome-scale assembly and evolution of the tetraploid *Salvia splendens* (Lamiaceae) genome. Horticulture Research, 8.

Kabir, M.H.; Mamun, A.N.K.; Roy, P.K.; Islam, M.R.; Jahan, M.T. and Talukder, S.U. (2014). In vitro mass propagation of salvia (*Salvia splendens*) from nodal explant. Nuclear Science and Applications, 23: 51-54.

Kalia, R.K.; Rai, M.K.; Kalia, S.; Singh, R. and Dhawan, A.K. (2011). Microsatellite markers: an overview of the recent progress in plants. Euphytica, 177(3): 309-334.

Karabacak, E.; Uysal, İ. and Dogan, M. (2009). Cultivated Salvia species in Turkey. Biyolojik Çeşitlilik ve Koruma, *2*(1), 71-77.

Karousou, R.; Hanlidou, E. and Kokkini, S. (2000). II. Botany the sage plants of greece: distribution and infraspecific variation. Sage: the genus Salvia, Harwood Academic Publishers, Amsterdam, The Netherlands, 27-46.

Khan, S.; Saeed, B. and Kauser, N. (2011). Establishment of genetic fidelity of in vitro raised banana plantlets. Pak J Bot, 43(1): 233-242.

Kılınç, F.M.; Suzerer, V.; Çiftçi, Y.O.; Onay, A.; Yıldırım, H.; Uncuoglu, A.A. and Metin, O.K. (2015). Clonal micropropagation of *Pistacia lentiscus* L. and assessment of genetic stability using IRAP markers. Plant growth regulation, 75: 75-88.

Krishna, H.; Alizadeh, M.; Singh, D.; Singh, U.; Chauhan, N.; Eftekhari, M. and Sadh, R. K. (2016). Somaclonal variations and their applications in horticultural crops improvement. Biotech, 6, 1-18.

Kumar, P. (2011). *Salvia splendens* (Scarlet sage). LAP Lambert Academic Publishing, ISBN 3844311904.

Landey Bobadilla, R.; Cenci, A.; Guyot, R.; Bertrand, B.; Georget, F.; Dechamp, E. and Etienne, H. (2015). Assessment of genetic and epigenetic changes during cell culture ageing and relations with somaclonal variation in *Coffea arabica*. Plant Cell, Tissue and Organ Culture (PCTOC), 122: 517-531.

Larkin, P.J. and Scowcroft W.R. (1981) Somaclonal variation a new source of variability from cell cultures for crop improvement. Theor Appl Genet 60:197–214.

Lázaro-Castellanos, J.O.; Mata-Rosas, M.; González, D.; Arias, S. and Reverchon, F. (2018). In vitro propagation of endangered Mammillaria genus (Cactaceae) species and genetic stability assessment using SSR markers. In Vitro Cellular & Developmental Biology-Plant, 54: 518-529.

Lee, T. (1987). Micropropagation of sugarcane (*Saccharum spp*.). Plant Cell, Tissue & Organ Culture, 10(1):47–55.

Mallón, R.; Rodríguez-Oubina, J. and González, M.L. (2011). Shoot regeneration from in vitro-derived leaf and root explants of *Centaurea ultreiae*. Plant Cell, Tissue and Organ Culture (PCTOC), 106: 523-530.

Maruthi, R.A.; Kumar, I.S. and Kishor, P.K. (2012). Effect of growth regulators and physiological gradients on the high frequency plant regeneration from the long-term callus cultures of different germplasms of rice *(Oryza sativa L.*). *Journal of Phytology*, *4*(2): 6–15.

Martínez-Estrada, E.; Caamal-Velázquez, J.H.; Salinas-Ruíz, J. and Bello-Bello, J.J. (2017). Assessment of somaclonal variation during sugarcane micropropagation in

temporary immersion bioreactors by intersimple sequence repeat (ISSR) markers. In Vitro Cellular & Developmental Biology-Plant, 53:553-560.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15: 473-97.

Murray, M.G. and Thompson, W.F. (1980). Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res., 8 (19): 4321-4325.

Neelakandan, A.K. and Wang, K. (2012). Recent progress in the understanding of tissue culture-induced genome level changes in plants and potential applications. Plant cell reports, *31*: 597-620.

Nei, M. (1987). Molecular evolutionary genetics. Columbia university press. New York.

Noormohammadi, Z.; Habibollahi, H.; Farahani, F. and Sheidai, M. (2020). Molecular analysis of somaclonal variation *in Linum usitatissimum and L. album*. Plant Gene, 24: 100261.

Papafotiou, M.; Vlachou, G. and Martini, A.N. (2023). Investigation of the Effects of the Explant Type and Different Plant Growth Regulators on Micropropagation of Five Mediterranean Salvia spp. Native to Greece. Horticulturae, *9*(1): 96.

Park, J.S.; Park, J.H.; Kim, S.J. and Park, Y.D. (2020). Genome analysis of tissue culture-derived variations in regenerated Brassica rapa ssp. pekinensis plants using next-generation sequencing. Horticulture, Environment, and Biotechnology, *61*: 549-558.

Pastelín Solano, M.C.; Salinas Ruíz, J.; González Arnao, M.T.; Castaneda Castro, O.; Galindo Tovar, M.E. and Bello Bello, J.J. (2019). Evaluation of in vitro shoot multiplication and ISSR marker based assessment of somaclonal variants at different subcultures of vanilla *(Vanilla planifolia Jacks)*. Physiology and Molecular Biology of Plants, 25: 561-567.

Peng, X., Zhang, T.T. and Zhang, J. (2015). Effect of subculture times on genetic fidelity, endogenous hormone level and pharmaceutical potential of *Tetrastigma hemsleyanum* callus. Plant Cell, Tissue and Organ Culture (PCTOC), 122: 67-77.

Posmyk, M.M. and Szafranska, K. (2016). Biostimulators: a new trend towards solving an old problem. Frontiers in plant science, 7: 1-6.

Prevost, A. and Wilkinson, M.J. (1999). A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. Theoretical and applied Genetics, 98: 107-112.

Ranghoo-Sanmukhiya, V.M. (2021). Somaclonal variation and methods used for its detection. Propagation and genetic manipulation of plants, 1-18.

Rai, M.K. (2022). Somaclonal variation in improvement of agricultural crops: recent progress. Agricultural biotechnology: latest research and trends, 129-146.

Rai, M.K. (2023). Start codon targeted (SCoT) polymorphism marker in plant genome analysis: current status and prospects. Planta, 257(2): 34.

Rai, M.K.; Kalia, R.K.; Singh, R.; Gangola, M.P. and Dhawan, A.K. (2011). Developing stress tolerant plants through in vitro selection—an overview of the recent progress. Environmental and experimental botany, 71(1): 89-98.

Rai, M.K.; Phulwaria, M.; Harish, Gupta, A.K.; Shekhawat, N.S. and Jaiswal, U. (2012). Genetic homogeneity of guava plants derived from somatic embryogenesis using SSR and ISSR markers. Plant Cell, Tissue and Organ Culture *(PCTOC*), 111: 259-264.

Rani, V. and Raina, S.N. (2000). Genetic fidelity of organized meristem-derived micropropagated plants: a critical reappraisal. In Vitro Cellular & Developmental Biology-Plant, 36: 319-330.

Rathore, N.S.; Rai, M.K.; Phulwaria, M.; Rathore, N. and Shekhawat, N.S. (2014). Genetic stability in micropropagated *Cleome gynandra* revealed by SCoT analysis. Acta physiologiae plantarum, 36: 555-559.

Regnault-Roger, C. (1997). The potential of botanical essential oils for insect pest control. Integrated Pest Management Reviews, 2: 25-34.

Roldan-Ruiz, I.; Dendauw, J.; Van Bockstaele, E.; Depicker, A. and De Loose, M.A.F. L. P. (2000). AFLP markers reveal high polymorphic rates in ryegrasses (*Lolium spp*.). Molecular breeding, 6:125-134.

Sales, E.K. and Butardo, N.G. (2014). Molecular analysis of somaclonal variation in tissue culture derived bananas using MSAP and SSR markers. International Journal of Biotechnology and Bioengineering, 8(6): 615-622.

Samarfard, S.; Kadir, M.A.; Kadzimin, S.B.; Saud, H.M.; Ravanfar, S.A. and Danaee, M. (2014). In vitro propagation and detection of somaclonal variation in *Phalaenopsis gigantea* as affected by chitosan and thidiazuron combinations. HortScience, 49(1): 82-88.

Satya, P.; Karan, M.; Jana, S.; Mitra, S.; Sharma, A.; Karmakar, P.G. and Ray, D.P. (2015). Start codon targeted (SCoT) polymorphism reveals genetic diversity in wild and domesticated populations of ramie (*Boehmeria nivea L. Gaudich.)*, a premium textile fiber producing species. Meta gene, *3*: 62-70.

Shalan, E.E.; Soliman, S.S.; Mahmoud, A.A.; Al-Khayri, J.M.; ALshamrani, S.M.; Safhi, F.A. and Hassanin, A.A. (2023). Micropropagation of Daylily *(Hemerocallis fulva)* from Crown-Tip Explants and Assessment of Somaclonal Variation of in Vitro-Propagated Plants Using SCoT Markers. Phyton (0031-9457), 92(7).

Shanker, D. and Unnikrishnan, P.N. (2001). An overview: Introduction to herbal plants used in medicine. Amruth, 5(4): 9-16.

Sharma, S.; Shahzad, A.; Kumar, J. and Anis, M. (2014). In vitro propagation and synseed production of scarlet salvia (Salvia splendens). Rendiconti Lincei, 25: 359-368.

Smulders, M.J.M. and De Klerk, G.J. (2011). Epigenetics in plant tissue culture. Plant growth regulation, 63: 137-146.

Stepankova, J. (2001). Clebsch, B.: A Book of Salvias: Sages for Every Garden. Biologia Plantarum, 1(44): 88-88.

Soliman, H.I.A.; Metwali, E.M.R. and Almaghrabi, O.A.H. (2014). Micropropagation of Stevia rebaudiana Betroni and assessment of genetic stability of in vitro regenerated plants using inter simple sequence repeat (ISSR) marker. Plant biotechnology, 31(3): 249-256.

Soundararajan, P.; Sivanesan, I.; Jo, E.H. and Jeong, B.R. (2013). Silicon promotes shoot proliferation and shoot growth of *Salvia splendens* under salt stress in vitro. Horticulture, Environment, and Biotechnology, 54: 311-318.

Tikendra, L.; Potshangbam, A.M.; Dey, A.; Devi, T.R.; Sahoo, M.R. and Nongdam, P. (2021). RAPD, ISSR, and SCoT markers based genetic stability assessment of micropropagated *Dendrobium fimbriatum Lindl. var. oculatum Hk*. f.-an important endangered orchid. Physiology and Molecular Biology of Plants, 27: 341-357.

Varshney, R.K.; Chabane, K.; Hendre, P.S.; Aggarwal, R.K. and Graner, A. (2007). Comparative assessment of EST-SSR, EST-SNP and AFLP markers for evaluation of genetic diversity and conservation of genetic resources using wild, cultivated and elite barleys. Plant Science, 173(6) :638-649.

Wu, D.L., Hou, S.W., Qian, P.P., Sun, L.D., Zhang, Y.C., and Li, W.J. (2009). Flower color chimera and abnormal leaf mutants induced by 12C6+ heavy ions in *Salvia splendens* Ker-Gawl. Scientia horticulturae, 121(4), 462-467.

Yu, L.; Chen, H.; Hong, P.; Wang, H. and Liu, K. (2015). Adventitious bud induction and plant regeneration from stem nodes of *Salvia splendens* 'Cailinghong'. Hortscience, 50(6): 869-872.

تقييـــم الاختلافات الوراثية لنبات السالفيا الحمراء عند دورات إكثار مختلفة باستخدام الواسمات الجزيئية

مني أحمـد طـه[1]، عزة عبـد العزيز توفيق[1]، عادل محمـد محمـود[2]، محمـد فؤاد محمـد[3]

[1]قسم الزينة، كلية الزراعة، جامعة أسيوط، أسيوط، مصر.
[2]قسم المحاصيل، كلية الزراعة، جامعة اسيوط، اسيوط، مصر.
[3]قسم الخضر، كلية الزراعة، جامعة أسيوط، مصر.

الملخص

يوفر التباين الوراثي الناتج عن زراعة الخلايا والأنسجة مصدرًا جديدًا للتنوع لتحسين أنواع نباتات الزينة. لذلك كان الهدف دراسة تأثير الثيديازيرون (TDZ) على تحفيز إنتاج الكالس وكذلك تم دراسة عدد دورات النقل الي البيئات والإكثار لنبات السالفيا الحمراء.

وقد أشـارت النتائج إلى أن الوسـط المغذي الذي يحتوي على TDZ بتركيز 0.5 ملجم/لتر مع IAA بتركيز 0.1 ملجم/لتر أدى إلى أعلى نسبة لإنتاج الكالس (60%).

بالنسـبة لتضـاعف الافرع، تم الحصـول على أفضـل النتائج عندما تمت زراعة قمة الفرع على بيئة (MS) موراشيج وسكوج المضاف إليها الثيديازيرون TDZ بتركيز 0.2 ملجم/لتر .

بالنســبة لدورات الإكثار، تم اســتخدام نفس تركيزات TDZ عند 0.2 ملجم / لتر وتم نقل الافرع القمية لعدد إحدى عشــر (11) دورة إكثار على فترات 21 يومًا خلال فترة إجمالية قدرها 231 يومًا.

للكشف عن الاختلافات الوراثية، تم استخدام الواسم من النوع SCoT وتم استخدام خمس بادئات SCoT وتم الحصول على إجمالي 24 حزمه. أظهرت النتائج وجود تعدد أشكال عالي بين النباتات الأم ونباتاتها المتجددة من دورة الإكثار رقم (11). أظهرت الواســمات عن تعدد أشــكال الذي يظهر أنماطًا مختلفة بشكل واضح لشظايا الحمض النووي، وقد لوحظت بعض الحزم الجديدة في النباتات المتجددة في جميع الواسمات الجزيئية التي تمت دراستها.

بالنســبة للتجذير، لوحظ أن أفضــل نســبة تجذير (100 %)، وأعلى عدد للجذور/ فرع (7.00) وطول الجذر (2.58 سم) عند زراعة الأفرع في وسط موراشيج وسكوج (MS) خالي من الهرمونات والمضــاف له 15 جرام ســكروز/لتر. وقد تم أقلمة النباتات بنجاح باســتخدام بيئة تحتوي علي البيتموس + البيرلايت (1: 1 علي اساس الحجم) بمعدل بقاء 100 %.