Industrial Crops & Products 187 (2022) 115404

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

High-frequency plant regeneration and genetic fidelity assessment of regenerants by molecular and biochemical markers in Woodland Sage (*Salvia nemorosa* L.)

Hamid Reza Heydari, Esmaeil Chamani *

Department of Horticultural Science, Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran

ARTICLE INFO

Keywords:
Cytokinin
Genetic Fidelity
In vitro Regeneration
Rosmarinic acid
Woodland Sage

ABSTRACT

Salvia nemorosa L. is a valuable medicinal and ornamental plant from Lamiaceae. A high-frequency protocol for in vitro regeneration from *S. nemorosa* leaf explants was developed based on our results. Various plant growth regulators were evaluated for their effects on direct and indirect organogenesis, callogenesis, shoot multiplication, and rooting of explants at different concentrations. The maximum percentage of direct shoot regeneration (97.5%) and the number of shoots per explant (8.6) were obtained on MS medium supplement with 8 μM TDZ plus 8 μM NAA. The result revealed that media amended by 16 μM 2, 4-D with 8 or 16 μM BA was the best treatment for callus induction. Media containing the combination of 8 μM TDZ plus 2 μM NAA provided the highest rate of shoot induction (84%) and the highest number of shoots (5.4) from calli. Regenerated shoots were multiplied efficiently with an average of 7.4 shoots and 32.4 leaves per explant on media containing 12 μM TDZ. In the present study, TDZ and BA treatments led to the highest proliferation rate; however, adenine sulfate was preferred for proliferation because it did not produce hyperhydric plants. The genetic fidelity of 19 plantlets (one donor plant, nine direct and nine indirect regenerants) was appraised by 10 ISSR markers. The molecular analysis revealed that direct organogenesis produced true-to-type plantlets and could be employed for clonal multiplication of Woodland Sage. Indirect regenerants showed polymorphic bands (12.5–33.33%), confirming the somaclonal variation occurrence during indirect organogenesis. Also, HPLC analysis of regenerants showed that two indirect regenerants accumulated higher rosmarinic acid content than mother plants, while three of them accumulated lower rosmarinic acid content. As part of this study, molecular and biochemical markers have been employed to investigate somaclonal variation in in vitro regeneration of the *Salvia* genus for the first time, which could be utilized to develop reliable methods for multiplying elite lines and achieving high-yield lines.

1. Introduction

Salvia is the largest genus of the Lamiaceae family, with over 1000 species distributed worldwide (Marchev et al., 2014). Members of this genus are a rich source of secondary metabolites such as terpenoids, sterols, flavonoids, and phenolic compounds (Wu et al., 2016). Additionally, extracts of Sage species represent antibacterial, antiviral, antifungal, anti-mutagenic, and anti-inflammatory activities (Jan et al., 2015). These properties caused the widespread use of *Salvia* species in the folk and modern medicinal, cosmetics, and pharmaceutical drugs industry and as a food additive (Wu et al., 2016).

Salvia nemorosa L. (Woodland Sage) is one of seventeen endemic *Salvia* species in Iran, which grows wild throughout its mountainous regions (Heydari et al., 2020a; Mirza and Sefidkon, 1999). This species is also distributed in central and southeastern Europe, southern Siberia, Caucasia, and Turkey (Kuźma and Wysokińska, 2003; Skała and Wysokińska, 2004). Woodland Sage is a hardy herbaceous perennial plant with high ornamental and medicinal value and uses widely in the garden and landscape design (Kaprinyak and Fari, 2019; Mirza and Sefidkon, 1999). In Turkish folk medicine, *S. nemorosa* leaves are traditionally applied externally to stop bleeding (Skała and Wysokińska, 2004). In Bulgarian traditional medicine, Woodland Sage is used mainly to treat

Abbreviations: 2, 4-D, 2, 4-Dichlorophenoxyacetic acid; AdS, Adenine Sulphate; BA, N6-Benzyladenine; CTAB, Cetyltrimethylammonium Bromide; IAA, Indole-3-Acetic Acid; IBA, Indole-3-butyric acid; KIN, Kinetin; mMS, Modified Murashige and Skoog; NAA, α-Naphthalene acetic acid; RA, Rosmarinic Acid.

* Corresponding author.
E-mail address: echamani@uma.ac.ir (E. Chamani).

https://doi.org/10.1016/j.indcrop.2022.115404
Received 16 April 2022; Received in revised form 14 July 2022; Accepted 19 July 2022
0926-6690/© 2022 Elsevier B.V. All rights reserved.

hemorrhages, stomach aches, furuncles, and diarrhea (Bahadori et al., 2017). This plant has been employed in traditional Iranian medicine to reduce pain and inflammation. Aqueous and ethanolic extracts from the aerial parts showed anti-nociceptive activity with an efficacy similar to classical analgesic drugs (Heydari et al., 2020a; Nikolova and Aneva, 2017). Aerial parts of Woodland Sage are a rich source of phenolic compounds. Rosmarinic acid (RA) is the most abundant phenolic compound detected in this plant (Bahadori et al., 2017). Rosmarinic acid is a phenolic molecule with various applications, varying from food preservatives to cosmetics, and is also a molecule of interest to the pharmaceutical industry (Marchev et al., 2021). The RA possesses anti-inflammatory, anti-oxidative, antibacterial, antiviral, anti-hyperglycemic, anticancer, hepatoprotective, analgesic, cardioprotective, and neuroprotective properties. It is used in modern medicine to treat Alzheimer's disease, diabetes, fibrosis, and various types of cancer such as colorectal, breast, pancreatic, and lung cancers (Hitl et al., 2021; Trócsányi et al., 2020). Salvianolic acids, which have been used to treat cardiovascular disorders, fibrosis, and cancer, are another significant phenolic compound isolated from *S. nemorosa* (Heydari et al., 2020b). Other phenolic compounds detected in *S. nemorosa* aerial parts include caffeic acid, chlorogenic acid, ferulic acid, syringic acid, and p-coumaric acid (Bahadori et al., 2017).

An efficient tissue culture protocol is valid when it produces true-to-type plants (Viehmannova et al., 2016). *In-vitro* condition is very stressful and may lead to genetic and chromosomal instabilities, called somaclonal variation. Although this phenomenon is useful for some breeding purposes, it induces variations that may not be desirable when the goal is clonal multiplication (Krishna et al., 2016). Somaclonal variation, on the other hand, has the potential to induce favorable characteristics and increase variation in a population. It has become a helpful tool in plant improvement with regard to obtaining variants with resistance to abiotic stresses, increased yield and quality, novel phenotypic traits, and aesthetic qualities (Pawełkowicz et al., 2021). Therefore, concerning the desired objectives, it seems necessary to find out whether somaclonal variation occurs during *in vitro* propagation of plants (Wang and Wang, 2012). Recently, polymerase chain reaction (PCR) techniques, particularly molecular markers, have been routinely used to assess genetic fidelity or somaclonal variation in regenerated plants during *in vitro* multiplication (Viehmannova et al., 2016). There are many techniques available, but the inter simple sequence repeat (ISSR) is advantageous because it eliminates many limitations of other techniques, like the high cost of AFLP, the requirement of species-specific primers for SSR, and the low reproducibility of RAPD (Martínez-Estrada et al., 2017; Peng et al., 2015). ISSR markers provide a highly polymorphic, simple, and fast procedure that incorporates most of the advantages of SSRs and AFLP to the universality of RAPD (Ulvrova et al., 2021). It has been utilized in many species (Dharman and Anilkumar, 2021; Pastelin Solano et al., 2019; Thakur et al., 2021; Ulvrova et al., 2021) to examine somaclonal variation among *in vitro* propagated plants, indicating that it is appropriate for validating genetic integrity after micropropagation (Pawełkowicz et al., 2021).

S.nemorosa is currently "underappreciated," but it could be a promising plant species in the future due to its high ornamental and medicinal values (Kaprinyak and Fari, 2019). Despite its valuable characteristics, it has received limited efforts to improve its growth, biochemical, and ornamental qualities (Heydari et al., 2020b). Plant breeders must compensate for these deficiencies by employing faster biotechnology methods such as somaclonal variation, gene cloning, metabolic pathway engineering, artificial polyploidy, etc. They lead to the generation of plants that vary in traits in a significantly shorter timeframe and with a reduced workload compared to conventional breeding methods, which take 10–15 years and are laborious (Niazian, 2019; Pawełkowicz et al., 2021). The tissue culture platform is the basis for most of the new biotechnological techniques employed in modern plant breeding. Therefore, establishing a reliable and efficient protocol for *in vitro* regeneration and micropropagation is necessary (Arora et al., 2022). Though the somaclonal variation in *in vitro* medicinal plant cultured is not well documented, one shall speculate that it is presumably involved in the regulating network of secondary metabolites production. Thus, its possible implication in triggering secondary metabolites production needs to be elucidated(Ngezahayo, 2018). According to our literature review, only one research by Skała and Wysokińska (2004) has been reported on the micropropagation of Woodland Sage. Only a few types and concentrations of plant growth regulators, particularly auxins, were examined in their study, and the genetic integrity of regenerated plants was not reported. Consequently, further studies must be conducted on the *in vitro* propagation of this plant, especially Iranian indigenous genotypes. Our study aimed to develop a reliable and efficient regeneration protocol through direct and indirect organogenesis. Then, *in-vitro* regenerants of *S. nemorosa* were subjected to ISSR marker analyses to assess genetic identity and investigate the occurrence probability of somaclonal variation. Finally, the amount of rosmarinic acid and Phenylalanine ammonia-lyase (PAL) enzyme activity were examined as biochemical markers to evaluate the occurrence of somaclonal variation in regenerated plants.

For the first time, molecular and biochemical markers have been employed in the present study to investigate somaclonal variations in *Salvia* species *in vitro* regeneration. This study is the first report on the validation of genetic fidelity in *S. nemorosa* regeneration. It could be used for large-scale multiplication of this species, which has excellent ornamental and medicinal potential.

2. Material and method

2.1. Plant material

Seeds of the Woodland Sage were gathered from Khansar, Isfahan province, which is located at 33° 10′ 00″ N, 50° 23′ 00″W. The seeds have been validated in the Herbarium of Research Institute of Forests and Rangelands, Tehran, Iran. They were subjected to a surface sterilization protocol of 70% (v/v) ethanol for 1 min and 2.5% chlorine bleach (NaClO) containing a few drops of Tween 20 for 15 min. After that, rinsed at 3, 5, and 15 min with sterile double distilled water and cultured in glass jars (200 mL) holding 25 mL Murashige and Skoog (MS) basal media (Murashige and Skoog, 1962) solidified by 5.8 g/l plant agar (Plant agar, Duchefa Biochemie, Netherlands). Then, they were placed under cool-white light illumination with a 16 h photoperiod at 24 ± 1 ° C. The seeds began germinating after 10 days of culture, and twenty-eight-day-old seedlings were employed as source explants. All explants employed in this study were taken from a single randomly chosen seedling to standardize the experiment. For this purpose, the leaves of the seedling were excised, and the shoots were transferred to a proliferation media described by Skała and Wysokińska (2004), which was MS medium containing 8.9 μM N6-Benzyladenine (BA) and 2.9 μM Indole-3-acetic acid (IAA). Shoot proliferation on the same medium was done until enough individual explants were obtained. In the final step, before the organization of trials, previously gained plantlets were sub-cultured on a MS medium without PGRs. Twenty-one days after the last subculture, mature leaves were cut and used as explant in direct and indirect regeneration experiments.

2.2. Effect of PGRs on callus induction and direct regeneration

The goal of the first experiment was to appraise the effect of various plant growth regulators (PGRs) on callus induction and direct regeneration in the leaves of *S. nemorosa*. For this purpose, 1 × 1 cm pieces of *in-vitro* raised leaves as an explant were transferred to the modified MS medium supplemented with BA and Thidiazuron (TDZ) as cytokinin and α-Naphthalene acetic acid (NAA) and 2,4-Dichlorophenoxyacetic acid (2, 4-D) as auxin. Our earlier tests (data not shown) indicated that a modified MS medium (MS basal salts amended with B5 vitamin mixture) was more suitable for callogenesis and organogenesis in Woodland Sage

than MS medium. Also, 100 mg/l of ascorbic acid was added to all media to prevent medium and explants browning by phenol secretion. Thirty-five PGR combinations included BA or TDZ at 0, 4, and 8 μM along with NAA or 2, 4-D at 0, 4, 8, and 16 μM used in this experiment (Table 1). All cultures were kept in the growth chamber under the dark condition at 24 ± 1 ºC for 4 weeks. During this period, cultures were monitored regularly, and the data was recorded. The experiment was done based on a completely randomized design with five replications (glass jars). Eight explants were placed per each replication (glass jars). All PGRs used in this study were purchased from Duchefa Biochemie, Netherlands.

2.3. Indirect regeneration

The second experiment was conducted to study the effect of PGRs on shoot regeneration from the callus of *S. nemorosa*. Calli was obtained in the first experiment, sub-cultured three times with 14-day intervals on mMS media supplemented by 16 μM 2, 4-D + 8 μM BA for proliferation. Then, 1 g of proliferated calli served as an explant in the current experiment. These explants were placed on mMS medium amended by BA or TDZ at 0, 4, 8, and 16 μM in combination with NAA at 0, 2, and 4 μM. All cultures were placed under the dark condition in the growth chamber at 24 ± 1ºC for 6 weeks. During this period, cultures were monitored regularly, and data was recorded. A completely randomized design with five replications (glass jars) was adopted for this experiment. Five explants were cultured per each replication (glass jars).

2.4. Shoot multiplication

The third experiment evaluated the influence of various types and concentrations of cytokinins on shoot multiplication of *S. nemorosa*. Shoots regenerated in previous experiments were cultured on mMS medium without PGRs for 28 days. These shoots were employed as the source of explants in the proliferation trial. At first, the leaves are excised, and then the shoots with a length of approximately 3 cm contain three nodes cultured on mMS medium supplemented by BA, TDZ, Kinetin (KIN), and adenine sulphate(AdS) at 0, 4, 8, and 12 μM with or without 2 μM NAA for 4 weeks. The experiment was done with five replications in a completely randomized design and five explants incubated in each replication. All cultured media were placed under cool-white light illumination with 16 h photoperiod at 24 ± 1 ºC. In this experiment, multiplied plantlets with a glassy appearance and brittle, translucent, and wrinkled leaves were characterized as hyperhydric shoots based on Gao et al. (2017).

2.5. Rooting and acclimatization

In a fourth experiment, some auxins were administered to regenerated shoots to observe their effects on root formation. 2–3 cm basal end of proliferated and elongated shoots were excised and transferred to root induction media which was constructed of ½ mMS media supplemented with IAA, NAA, and IBA (Indole-3-butyric acid) at 0, 2, 4, 6, and 8 μM plus 500 mg/l activate charcoal and 100 mg/l ascorbic acid. The experiment was conducted on a completely randomized design with five replications (glass jars). In each replication, 10 explants were cultured.

Table 1
Effect of plant growth regulators on organogenesis and callogenesis of *S. nemorosa* from leave explants.

Number	NAA	Plant Growth Regulators (μM)			Callus Initiation (%)	Day to Callus Initiation	Callus Fresh Weight (g)	Regeneration (%)	Day to Regeneration Initiation	Number of Regenerated Shoots
		2, 4-D	BA	TDZ						
1	0	0	0	0	0.0 ± 0.0^{h}	0.0 ± 0.0^{i}	0.0 ± 0.0^{l}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
2			4		0.0 ± 0.0^{h}	0.0 ± 0.0^{i}	0.0 ± 0.0^{l}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
3			8		0.0 ± 0.0^{h}	0.0 ± 0.0^{i}	0.0 ± 0.0^{l}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
4				4	0.0 ± 0.0^{h}	0.0 ± 0.0^{i}	0.0 ± 0.0^{l}	37.5 ± 5.5^{f}	16.2 ± 0.58^{bc}	2.4 ± 0.50^{g}
5				8	0.0 ± 0.0^{h}	0.0 ± 0.0^{i}	0.0 ± 0.0^{l}	50 ± 3.9^{e}	15.2 ± 0.37^{cd}	4.2 ± 0.20^{f}
6	4				25.0 ± 3.9^{g}	15.2 ± 0.8^{a}	0.11 ± 0.003^{k}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
7	8				35.0 ± 4.6^{f}	14.4 ± 0.5^{ab}	0.13 ± 0.005^{j}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
8	16				62.5 ± 3.9^{d}	13.8 ± 0.7^{b}	0.14 ± 0.006^{i}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
9	4		4		0.0 ± 0.0^{h}	0.0 ± 0.0^{i}	0.0 ± 0.0^{ll}	37.5 ± 5.5^{f}	18.8 ± 0.73^{a}	2.4 ± 0.50^{g}
10	8		4		0.0 ± 0.0^{h}	0.0 ± 0.0^{i}	0.0 ± 0.0^{l}	52.5 ± 7.2^{e}	18.4 ± 0.67^{a}	10 ± 0.44^{g}
11	16		4		65.0 ± 7.2^{d}	11.2 ± 0.3^{de}	0.19 ± 0.003^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
12	4		8		42.5 ± 3.0^{ef}	13.4 ± 0.7^{b}	0.15 ± 0.003^{i}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
13	8		8		0.0 ± 0.0^{h}	0.0 ± 0.0^{i}	0.0 ± 0.0^{l}	52.5 ± 4.6^{e}	17 ± 0.70^{b}	5.2 ± 0.58^{e}
14	16		8		0.0 ± 0.0^{h}	0.0 ± 0.0^{i}	0.0 ± 0.0^{l}	60 ± 6.1^{de}	14.2 ± 0.58^{bc}	4.6 ± 0.50^{ef}
15		4			37.5 ± 5.5^{f}	13.4 ± 0.6^{b}	0.13 ± 0.005^{j}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
16		8			50.0 ± 3.2^{e}	11.2 ± 0.6^{de}	0.15 ± 0.003^{i}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
17		16			77.5 ± 8.2^{c}	10.6 ± 0.6^{def}	0.17 ± 0.007^{h}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
18		4	4		47.5 ± 2.5^{e}	12.0 ± 0.7^{cd}	0.14 ± 0.005^{ij}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
19		8	4		85.0 ± 4.6^{bc}	11.2 ± 0.3^{de}	0.19 ± 0.003^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
20		16	4		100 ± 0.0^{a}	8.8 ± 0.3^{gh}	0.26 ± 0.007^{cd}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
21		4	8		50.0 ± 3.9^{e}	11.2 ± 0.5^{de}	0.17 ± 0.007^{h}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
22		8	8		87.5 ± 5.5^{b}	9.8 ± 0.3^{efg}	0.23 ± 0.005^{e}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
23		16	8		100 ± 0.0^{a}	8.4 ± 0.3^{h}	0.27 ± 0.014^{c}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
24	4			4	0.0 ± 0.0^{h}	0.0 ± 0.0^{i}	0.0 ± 0.0^{l}	65 ± 6.1^{cd}	14.2 ± 0.58^{bc}	4 ± 0.32^{f}
25	8			4	0.0 ± 0.0^{h}	0.0 ± 0.0^{i}	0.0 ± 0.0^{l}	72.5 ± 6.1^{c}	15.6 ± 0.68^{cd}	6.2 ± 0.58d
26	16			4	60.0 ± 4.6^{d}	11.0 ± 0.2^{de}	0.26 ± 0.007^{d}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
27	4			8	0.0 ± 0.0^{h}	0.0 ± 0.0^{i}	0.0 ± 0.0^{l}	95 ± 3.0^{a}	13.4 ± 0.74^{f}	8.0 ± 0.63^{ab}
28	8			8	0.0 ± 0.0^{h}	0.0 ± 0.0^{i}	0.0 ± 0.0^{l}	97.5 ± 2.5^{a}	13.8 ± 0.58^{ef}	8.6 ± 0.67^{a}
29	16			8	0.0 ± 0.0^{h}	0.0 ± 0.0^{i}	0.0 ± 0.0^{l}	82.5 ± 5.1^{b}	14.6 ± 0.40^{de}	7.4 ± 0.67^{bc}
30		4		4	50.0 ± 3.9^{e}	13.2 ± 0.8^{bc}	0.18 ± 0.006^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
31		8		4	87.5 ± 5.5^{b}	10.6 ± 0.6^{def}	0.21 ± 0.004^{f}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
32		16		4	100 ± 0.0^{a}	9.8 ± 0.6^{gef}	0.29 ± 0.007^{b}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
33		4		8	0.0 ± 0.0^{h}	0.0 ± 0.0^{i}	0.0 ± 0.0^{l}	65 ± 6.1^{cd}	14.8 ± 0.37^{de}	6.6 ± 0.67^{cd}
34		8		8	0.0 ± 0.0^{h}	0.0 ± 0.0^{i}	0.0 ± 0.0^{l}	60 ± 6.1^{de}	15.6 ± 0.67^{cd}	6.2 ± 0.67d
35		16		8	100 ± 0.0^{a}	9.4 ± 0.4^{fgh}	0.32 ± 0.009^{a}	0.0 ± 0.0^{g}	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}

Mean ± SD values in a column, followed by different letters, were significantly different according to the Duncan's multiple range test ($P \leq 0.05$).

All cultures were incubated at 24 ± 1 °C for a 16 h photoperiod under cool-white light illumination. After 14-days, shoots were taken out of the agar medium, and data were recorded. After four weeks, the well-rooted plantlets (176 plantlets) were excided from the culture medium, and the agar was removed by rinsing with sterile distilled water. They were then transferred into plastic pots including cocopeat, peat moss, and perlite (2:1:1). A half strength of MS basal liquid was used to moisten the soil mixture, and pots were covered with transparent polythene bags to hold high humidity. Then, they were maintained in growth chambers at 24 ± 1 °C under a 16 h light and 8 h dark photoperiod at a photon flux rate of 60 μmol m-2 s-1 provided by cool fluorescent lamps. After 10 days the polythene bags were removed gradually from the pots and the acclimatized plantlets were finally transferred to the greenhouse.

2.5.1. Phenolic compounds extraction

The leaf and stem tissues were ground into a fine powder after being oven-dried for 48 h at 45 degrees Celsius until constant dry weight. 500 mg of powdered samples were mixed well with 10 cc methanol. The mixture was then sonicated at room temperature for 30 min. The extracts were then filtered using Whatman no. 1 filter paper. A rotary evaporator was used to remove the solvent from the extracts. Then, 10 mg of dried extract was dissolved in 10 mL methanol and kept for further analysis.

2.5.2. Quantification of rosmarinic acid

A high-performance liquid chromatography method was used to assess the content of rosmarinic acid (RA), according to Heydari et al. (2020b). The mobile phase consisted of methanol (solvent A) and distilled water (solvent B) with a 1 mL/min flow rate. Gradient elution was carried out in a following system: 0–30 min, 88% A, 12% B; 30–45 min, 80% A, 20% B; 45–60 min, 70% A, 30% B. The oven temperature was maintained at 25 °C, and peaks were monitored at 195–400 nm wavelength. For the calibration curve, 20–100 μl of the methanolic standard solution of rosmarinic acid was applied in triplicate onto the HPLC. The rosmarinic acid calibration curve was depicted by plotting peak area versus concentrations. Using the equation obtained from the calibration curve, the concentration of the unknown samples was determined. The rosmarinic acid chromatographic peak was recognized by comparing its retention time with its reference standards.

2.5.3. PAL activity

Phenylalanine ammonia-lyase (PAL) activity was measured as described by Dong et al. (2010). The leaf tissue (2 g) was homogenized with 5 mL of extraction buffer utilizing a mortar and pestle. The homogenate was filtrated through four layers of cheesecloth and centrifuged at 12,000g for 15 min at 4 ∘C. The supernatant was used as a crude enzyme. The reaction mixture (3 mL) contained 0.5 mL of the crude enzyme, 16 mM of L-phenylalanine, 50 mM of Tris HCl buffer (pH8.9), and 3.6 mM of NaCl. The mixture was incubated at 37 ∘C for 60 min and the reaction was stopped by 500 μl of 6 M HCl. The reaction mixture was then centrifuged for 10 min at 12,000×g. Production of cinnamic acid was measured as an increase in absorbance at 290 nm. The enzyme activity was calculated per unit weight of protein.

2.6. DNA isolation and ISSR analysis

For genetic fidelity studies, nine *in-vitro* raised plantlets through indirect regeneration, nine direct regenerated plantlets, and a control plant were chosen randomly. The modified CTAB method described by Aleksic et al. (2012) was used for the extraction of genomic DNA extraction from fresh leaves. The quality of the extracted DNA was certified in 0.8% agarose (Duchefa Biochemie, Netherlands) stained with 10 μl Gel Stain (Invitrogen, USA) and using a Nanodrop Spectrophotometer (Thermo Scientific, USA). All DNA samples were adjusted to the final concentration of 50 ng/μl for PCR and preserved in the refrigerator at − 20 ℃. Out of 18 tested primers (Integrated DNA Technologies, Belgium), 10 produced at least three clear and scorable bands, and these primers were used for further PCR analysis. The reaction mixture sets in the volume of 20 μl included 2.5 μl template DNA (50 ng/l), 1.5 μl primer, 6 μl sterile double-distilled H_2O, and 10 μl 2x Master Mix [150 mM Tris– HCl, pH 8.5, 40 mM $(NH_4)2SO4$, 0.2% Tween 20, 3 mM $MgCl_2$, 400 μM of each dNTPs, 100 U/mL Taq-Purple DNA polymerase, Inert red dye and stabilizer (AMPLIQON, Denmark)]. Amplifications were performed in a Q-Cycler Thermal Cycler (Bio-Rad, USA), programmed with an initial denaturation at 94 ℃ for 5 min, followed by 40 cycles of denaturation at 94 ℃ for 1 min; annealing temperatures ranged from 47 to 53 ℃ for 1 min, and extension at 72 ℃ for 2 min, with the final extension at 72 ℃ for 8 min. Amplicons were separated on 1.2% agarose gel stained by 10 μl Gel Stain and run at 75 V for about 1.2 h. The amplified stained fragments were visualized on an agarose gel in Gel Documentation System under UV illumination.

2.7. Statistical analysis

All experiments were done based on a completely randomized design with five replications. The SAS 9.4 software was used for analyzing the data. Duncan's multiple range test (DMRT) was served at the 5% probability level to compare the means. ISSR analysis was done by scoring the reproducible and clear bands based on their presence (1) or absence (0) in the agarose gel.

3. Results

3.1. Effect of PGRs on callus induction and direct regeneration

The different types and concentrations of PGRs and their combinations used for callus induction and shoot regeneration from leaf explants had significant ($P \leq 0.05$) effects on measured traits (Table 1). No responses were observed from explants incubated on PGRs free medium (control). After about a week of culture, most of the explants began to brown and yellow. However, some of them remained green without any morphogenesis response. It indicated that the presence of an appropriate plant growth regulator in the *S. nemorosa* culture medium was essential for the initiation of organogenesis and callus formation. Among the two applied auxins in this experiment, most treatments containing 2, 4-D resulted in callogenesis (Fig. 1-b). However, PGR combinations containing NAA showed different responses depending on the type and concentration of participant cytokinins. 2, 4-D alone and in combination with BA and TDZ at all levels except two combinations (4 μM 2, 4-D + 8 μM TDZ and 8 μM 2, 4-D + 8 μM TDZ treatments) resulted in callus induction. On the other hand, regeneration was achieved in media containing NAA except for treatments with a high ratio of NAA to cytokinins, which led to callus induction (16 μM NAA+ 4 μM TDZ and 16 μM NAA+ 4 μM BA). Out of the 35 combined treatments of PGRs used in these experiments, only 19 treatments led to callogenesis, which significantly ($P \leq 0.05$) differed. A week after culture, in some treatments, including high auxin levels, the explants began to swell and formed a callus on their cut edges (Fig. 1, a-b).

At the end of the culture period, white friable, creamy compact, and white compact calli were developed based on PGRs treatments. Although acceptable callus formation has been attained in the presence of NAA or 2, 4-D (4–16 μM) in the culture media, their combination with cytokinin (TDZ or BA) accelerated callus initiation and enhanced the percent of callus induction. The result showed that the highest callus induction frequency (100%) was obtained in media amended with 16 μM 2, 4-D + 4, and 8 μM BA and TDZ (Table 1). Results also revealed that callus formation and callus weight increased as cytokinin concentration increased. Our results showed that for callus initiation under *in-vitro* conditions, NAA was generally less effective than 2, 4-D. No significant ($P \leq 0.05$) difference was found in the case of different cytokinins (BA and TDZ) effects on morphogenetic response. However, their responses are dependent on the companion auxin. Both caused

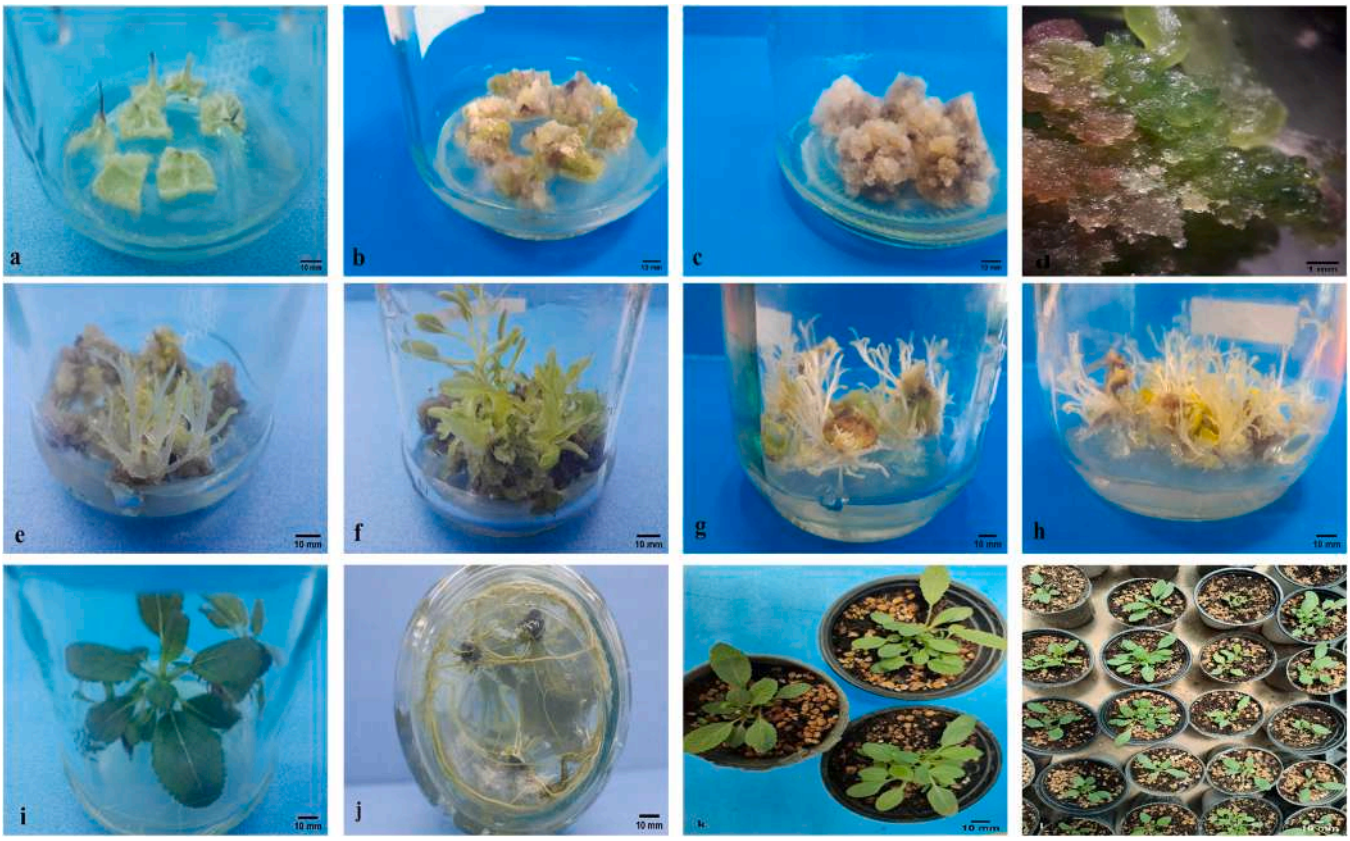

Fig. 1. - Morphogenesis in *S. nemorosa*. **a.** Callus initiated in cut edges of explants after 7 days from incubation; **b.** Callus formation at the end of experiment; **c.** Callus proliferation; **d.** Shoot primordia formation on calli; **e-f.** Indirect shoot regeneration; **g-h.** Direct shoot regeneration; **i-j.** Rooted plantlets; **k-l.** Acclimatized plant.

callogenesis in most combinations containing 2, 4-D, and induced organogenesis in NAA, including treatments. The results showed that TDZ alone induced adventitious shoots in explants (Fig. 1, g-h). However, BA was unable to develop any morphogenetic response without the presence of auxin. Overall, for direct *in-vitro* regeneration of *S. nemorosa,* TDZ showed more effective than BA. However, media containing BA and TDZ produced the highest number of new plantlets and shoot regeneration frequency, respectively. It's found that the greatest direct regeneration rate was 97.5% and 95% with 8 μM TDZ + 8 μM NAA and 8 μM TDZ + 16 μM NAA treatments, respectively. Moreover, the last PGRs combinations gave the highest shoot number (8.6 and 8 per explant) and the least time (13.8 and 14.6 days) to organogenesis initiation. In fact, the combination of BA with NAA in culture media led to the regeneration of a few number of shoots when compared to media containing TDZ.

3.2. Indirect regeneration

The result indicated that media without PGRs failed to regenerate any shoots from the callus, and the callus began to brown and eventually ruined two weeks after incubation. Adventitious shoot initiation started from the explants in other mediums after about 23 days (Fig. 1, d-f). The best results were obtained when callus pieces were placed on media amended with 8 μM TDZ + 2 μM NAA which induced the highest number of shoots (5.8) per explants and regeneration frequency (84%). TDZ produced higher shoots per explant than BA, according to the results (Table 2). It was also found that the result of TDZ treatment was better at lower concentrations and its optimum level was 8 μM. By increasing the TDZ concentration, the number of regenerated shoots decreased. On the other hand, increasing the BA concentration increased the regeneration rate, and its best concentration was 16 μM. The results of this experiment indicate that the presence of low doses of NAA in the regeneration media increased the adventitious shoot production in the presence of an appropriate ratio of cytokinin to auxin. It is concluded that in our experiment, the best result of PGRs combinations was the ratio of 4–1 cytokinin to auxin.

Table 2
Effect of plant growth regulators on organogenesis from calli in *S. nemorosa.*

Number	Plant Growth Regulators (μM)			Regeneration (%)	Number of Regenerated Shoots
	TDZ	BA	NAA		
1	0	0	0	0.0 ± 0.0^{h}	0.0 ± 0.0^{h}
2	4		0	44 ± 4.0^{efg}	$3.8 \pm 0.58^{c-f}$
3	8		0	64 ± 4.0^{bc}	4.6 ± 0.50^{abc}
4	16		0	48 ± 4.8^{def}	3 ± 0.31^{efg}
5		4	0	32 ± 4.8^{g}	2.6 ± 0.24^{fg}
6		8	0	44 ± 4.0^{efg}	$3.8 \pm 0.58^{c-f}$
7		16	0	52 ± 4.8^{cde}	4.0 ± 0.31b-e
8	4		2	68 ± 4.8^{b}	$4.2 \pm 0.37^{b-e}$
9	8		2	84 ± 4.0^{a}	5.6 ± 0.24^{a}
10	16		2	44 ± 4.0^{efg}	$3.6 \pm 0.40^{c-f}$
11		4	2	40 ± 6.3^{efg}	3 ± 0.40^{efg}
12		8	2	48 ± 4.8^{def}	$4.4 \pm 0.24^{a-d}$
13		16	2	60 ± 6.3^{bcd}	5.2 ± 0.58^{ab}
14	4		4	60 ± 6.3^{bcd}	$3.2 \pm 0.48^{d-g}$
15	8		4	84 ± 4.0^{a}	5.2 ± 0.20^{ab}
16	16		4	52 ± 4.8^{cde}	$4.2 \pm 0.58^{b-e}$
17		4	4	$36 \pm 4.0\ f^{g}$	2.2 ± 0.58^{g}
18		8	4	44 ± 4.0^{efg}	$4.4 \pm 0.24^{a-d}$
19		16	4	64 ± 4.0^{bc}	4.6 ± 0.50^{abc}

Mean ± SD values in a column, followed by different letters, were significantly different according to the Duncan's multiple range test ($P \leq 0.05$).

3.3. Shoot multiplication

In this experiment, different concentrations of TDZ, BA, KIN, and AdS (0, 4, 8, and 12 μM) were evaluated to attain strong and healthy shoots. A significant ($P \leq 0.05$) difference was observed between treatments. Regarding the number of regenerated shoots and leaves per each explant, TDZ showed a better response than others. However, shoot length was significantly ($P \leq 0.05$) more in KINand AdS supplemented media. New shoots began to form ten days after the explant establishment. After four weeks, media enriched with 12 μM TDZ produced the highest number of adventitious shoots (7.4) and leaves (32.4) per explant (Table 3). However, the high percentage of shoots (54%) grown on that media were shown hyperhydricity symptoms. Raising the concentration of TDZ and BA in the media from 4 to 12 μM increased the multiplication rate. However, the percentage of hyperhydricity (undesirable response) in media containing TDZ raised from 28% to 54% and BA from 22% to 46%. Media supplemented with adenine sulphate (4–12 μM) produced the highest shoot length (2.17–2.72 cm) without hyperhydricity (Fig. 1-i). Although the proliferation rate in media containing AdS is lower than TDZ and BA treatments, the appropriate shoot length was gained, and hyperhydricity did not occur. Hence, it seems the last one should be media for the multiplication of the Woodland Sage.

3.4. Rooting and acclimatization

The *in-vitro* grown shoots were transferred to the half-strength mMS medium containing IAA, NAA, and IBA at 0, 2, 4, 6, and 8 μM. The results revealed that all explants incubated in these treatments produced healthy roots (Fig. 1-j). The highest and significant ($P \leq 0.05$) root number per explants were obtained at 4 and 6 μM NAA (6.4, 6.2 respectively) and 6 μM IAA (6.2) treatments (Table 4). However, NAA at all concentrations provided the highest root number and length rather than IAA. However, media containing NAA (all concentrations) and IBA (only at 6 and 8 μM) formed a callus. Generally, three evaluated auxins showed a decrement in root number and length at 8 μM. However, it formed thickened roots. Overall, it has been concluded that IAA at the rate of 6 μm was the optimum concentration for the *in-vitro* rooting of *S. nemorosa*. *In vitro*, rooted plantlets were successfully acclimatized in a growth chamber and then transferred to the greenhouse. A total of 176 plantlets were transferred to the greenhouse for acclimatization, and the percentage of surviving plants was 94% after 12 weeks. Acclimatized plants appeared normal and did not exhibit any morphological abnormalities (Fig. 1, k-l).

3.5. ISSR analysis

Genetic stability investigation using genomic DNA of the donor and in-vitro raised plantlets from two regeneration methods was carried out to evaluate the possibility of the somaclonal variation using the ISSR marker. Nine randomly selected in-vitro propagated plantlets from organogenesis methods (direct and indirect organogenesis) were used and compared with the mother plant. A total of 1240 fragments of between 100 and 1100 bp were amplified using ISSR primers (Table 5). The number of bands per primer differed from four to eight, with an average of 6.4. In the case of the nine directly regenerants and mother plants, the banding patterns generated by ISSR primers are monomorphic, indicating no genetic variation within the samples. However, banding patterns among indirectly regenerated plantlets and donor plants revealed the occurrence of polymorphism. In this case, seven primers of total 10 primers namely UBC-807, UBC-809, UBC-810, UBC-811, UBC-834, UBC-843 and UBC-847 showed polymorphism in 12.5–33.33%. The primers that produced more polymorphic bands (2 bands) were UBC-807 and UBC-810, with 33.33% and 28.57%, respectively (Fig. 2).

3.6. Rosmarinic acid content and PAL activity

Rosmarinic acid (RA) content and phenylalanine ammonia-lyase (PAL) activity of Woodland Sage plantlets were measured at the end of culture using HPLC and spectrophotometric methods, respectively. As shown in Figs. 3 and 4, the activity of the PAL enzyme and the RA content in plantlets acquired from indirect regeneration were different. In contrast, the amount of these parameters in plantlets achieved from direct organogenesis was equal to the mother plant. HPLC analysis of 19 regenerants indicated that I2 and I6 regenerants had the highest RA

Table 4
Effect of Different Auxins on Rooting of explants in *S. nemorosa.*

	Plant Growth Regulators (μM)				
Number	NAA	IAA	IBA	Root Number	Root Length (cm)
1	0			0.00. ± 0.00^{d}	0.00 ± 0.00^{g}
2	2			5.00 ± 0.37^{b}	3.75 ± 0.14^{ef}
3	4			6.66 ± 0.60^{a}	4.69 ± 0.06^{ab}
4	6			6.50 ± 0.37^{a}	4.92 ± 0.05^{a}
5	8			4.66 ± 0.60^{b}	4.21 ± 0.05^{cd}
6		2		3.50 ± 0.37^{c}	3.57 ± 0.06^{f}
7		4		5.00 ± 0.37^{b}	4.40 ± 0.05^{bc}
8		6		6.50 ± 0.37^{a}	4.76 ± 0.08^{a}
9		8		5.33 ± 0.24^{ab}	4.39 ± 0.05^{bc}
10			2	3.50 ± 0.58^{c}	3.67 ± 0.4^{f}
11			4	5.66 ± 0.60^{ab}	4.43b± 0.04^{c}
12			6	4.50 ± 0.31^{b}	4.05 ± 0.05^{de}
13			8	6.00 ± 0.31^{a}	3.77 ± 0.08^{ef}

Mean ± SD values in a column, followed by different letters, were significantly different according to the Duncan's multiple range test ($P \leq 0.05$).

Table 3
Effect of plant growth regulators on proliferation of nodal explants in *S. nemorosa.*

		Plant Growth Regulators (μM)						
Number	TDZ	BA	KIN	AdS	Mean shoot number per explant	Mean shoot length (cm)	Mean number of leaves per explant	Hyperhydricity frequency (%)
1	0	0	0	0	1.4 ± 0.24^{g}	1.12 ± 0.06^{i}	6.6 ± 0.67^{g}	0.0 ± 0.0^{f}
2	4				5.0 ± 0.54^{bcd}	1.44 ± 0.04^{h}	23.2 ± 1.15^{cd}	28.0 ± 1.42^{c}
3	8				6.2 ± 0.37^{ab}	1.81 ± 0.14^{fg}	27.6 ± 1.07 ± ^{b}	42.0 ± 2.28^{b}
4	12				7.4 ± 0.50^{a}	2.1 ± 0.07^{cde}	32.4 ± 0.92^{a}	54.0 ± 2.28^{a}
5		4			3.2 ± 0.58^{ef}	1.62 ± 0.06^{gh}	15.2 ± 1.24^{f}	22.0 ± 2.28^{d}
6		8			4.4 ± 0.50^{cde}	1.83 ± 0.03^{efg}	22.8 ± 1.06^{cd}	30.0 ± 2.09^{c}
7		12			6.4 ± 0.50^{ab}	2.21 ± 0.06^{cd}	25.4 ± 0.050^{bc}	46.0 ± 3.16^{b}
8			4		2.8 ± 0.37^{efg}	1.94 ± 0.06^{def}	16.8 ± 0.60^{f}	0.0 ± 0.0^{f}
9			8		3.2 ± 0.37^{ef}	2.36 ± 0.05^{bc}	15 ± 0.63^{f}	10.0 ± 1.09^{e}
10			12		5.2 ± 0.80^{bc}	2.81 ± 0.18^{a}	20.6 ± 0.92^{de}	28.0 ± 1.42^{c}
11				4	2.6 ± 0.40^{fg}	2.17 ± 0.09^{cd}	13.4 ± 0.81^{f}	0.0 ± 0.0^{f}
12				8	3.6 ± 0.50^{def}	2.56 ± 0.14^{ab}	15.2 ± 0.80^{f}	0.0 ± 0.0^{f}
13				12	4.2 ± 0.58^{c-e}	2.72 ± 0.05^{a}	18.4 ± 0.81^{e}	0.0 ± 0.0^{f}

Mean ±SD values in a column, followed by different letters, were significantly different according to the Duncan's multiple range test ($P \leq 0.05$).

Table 5
List of ISSR primer sequences with the number and size of amplified fragments generated in direct and indirect organogenesis.

				Donor Plant	Direct Regeneration			Indirect Regeneration			
No.	Primers	Primer Sequence	Tm	nSB	nTSB	nTPB	P (%)	nSB	nPB	P (%)	Range (bp)
1	UBC-807	$(AG)_8T$	50	6	6	0	0	7	2	28.57	250–750
2	UBC-809	$(AG)_8G$	52	4	4	0	0	5	1	20	200–1000
3	UBC-810	$(GA)_8T$	50	6	6	0	0	6	2	33.33	100–800
4	UBC-811	$(GA)_8C$	52	5	5	0	0	5	1	20	250–800
5	UBC-826	$(AC)_8C$	52	5	5	0	0	5	0	0	200–900
6	UBC-828	$(TG)_8A$	50	8	8	0	0	8	0	0	100–900
7	UBC-834	$(AG)_8YT$	52	8	8	0	0	8	1	12.5	100–700
8	UBC-843	$(CT)_8RA$	48	7	7	0	0	7	1	14.28	300–800
9	UBC-847	$(CA)_8RC$	48	4	4	0	0	5	1	20	300–1100
10	UBC-860	$(TG)_8AA$	52	7	7	0	0	8	0	0	100–900

T_m: annealing temperature; **bp**: base pair; **R**= (A, G), **Y**= (C, T).

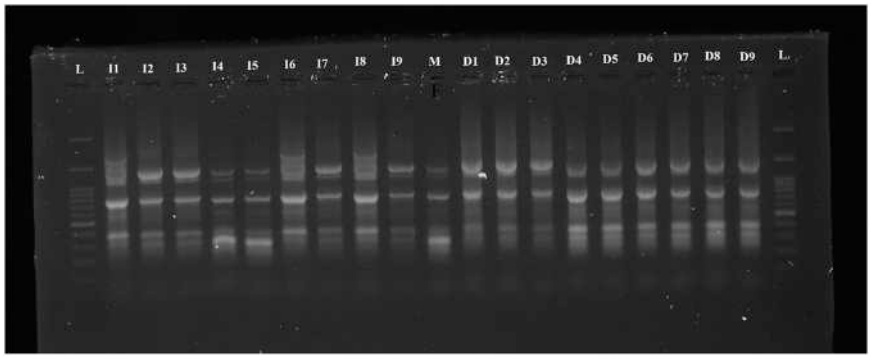

Fig. 2. - ISSR profiles of the mother plant (M), indirect regenerants (I1-I9) and direct regenerants (D1-D9) of *S. nemorosa* using UBC-847 primer.

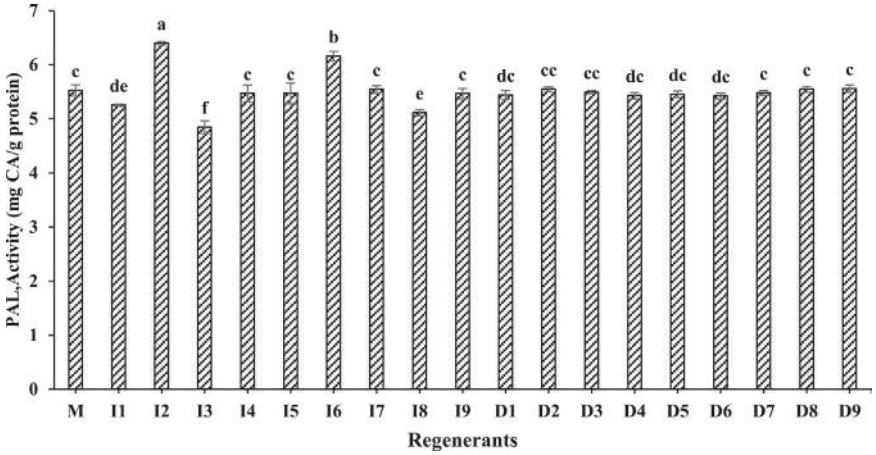

Fig. 3. - PAL activity of the mother plant (M), indirect regenerants (I1-I9) and direct regenerants (D1-D9) of *S. nemorosa.*

content, which was 9.18 and 8.81 mg/g DW, respectively (Fig. 5). Also, I8, I3, and I1 showed the lowest RA content with 6.30, 6.09, and 6.72, respectively. As shown in Fig. 5, cluster analysis grouped the regenerants into three categories, which include group 1 (I2 and I6), group 2 (I1, I3, and I8), and group 3 (I4, I5, I9, and D1–9) (Fig. 6).

4. Discussion

In the present study, direct and indirect organogenesis was induced from *in-vitro* derivative leaves of S. *nemorosa*. It is vital in two respects. Firstly, often true-to-type plants were obtained through direct organogenesis because of direct regeneration from explants (Bhusare et al., 2018). At least the occurrence of genetic changes is crucial in propagating elite genotypes and cultivars (Nayak et al., 2012; Viehmannova et al., 2016). Secondly, in the indirect regeneration via callus, somaclonal variation may occur. Although it is not desirable for true-to-type plant production, it can be used for genetic improvement and breeding purposes (Bairu et al., 2010; Miguel and Marum, 2011).

In the first experiment of the present research, thirty-five combinations of two cytokinins (BA and TDZ) and two auxins (NAA and 2, 4-D) assessed on organogenesis and callogenesis potential of leaf explants. Explants incubated on PGRs free medium could not stimulate any morphogenesis response. It indicated that the presence of an appropriate plant growth regulator in the *S. nemorosa* culture medium is essential for initiating organogenesis and callus formation. Similar results have been reported in *in-vitro* studies of other *Salvia* species such as *Salvia broussonetii* (Mederos-Molina, 2006), *Salvia canariensis* L. (Mederos-Molina, 2004) and *Salvia miltiorrhiza* (Tsai et al., 2015). Our findings revealed

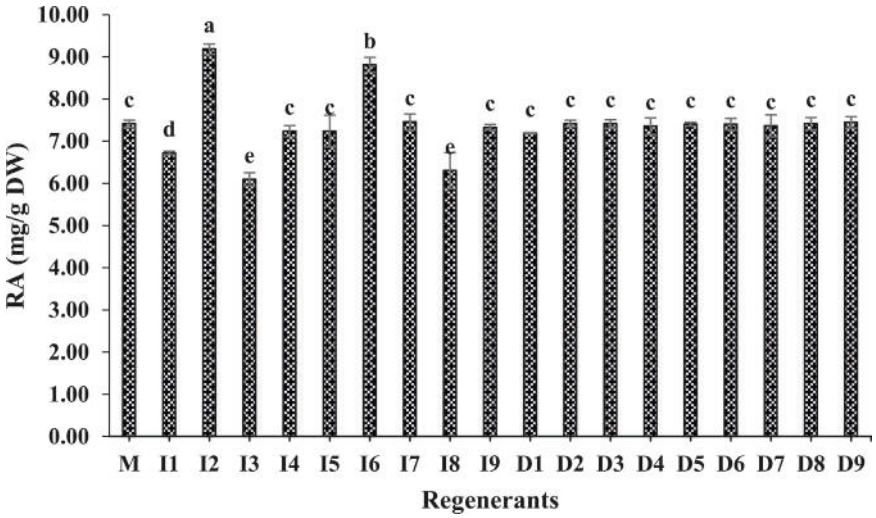

Fig. 4. - Rosmarinic acid content of the mother plant (M), indirect regenerants (I1-I9) and direct regenerants (D1-D9) of *S. nemorosa.*

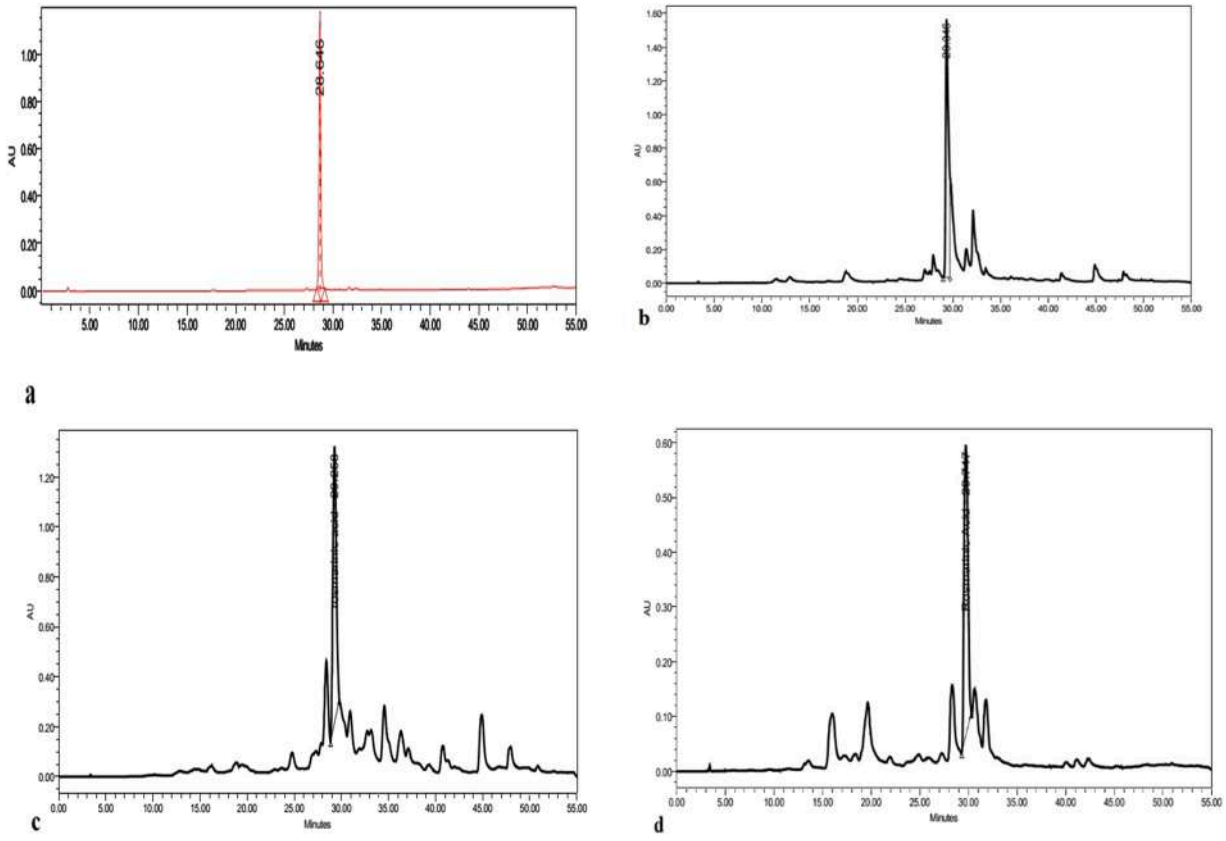

Fig. 5. - HPLC chromatograph of rosmarinic acid standard (a), I2 (b), mother plant (c) and I8 (d) regenerants.

that TDZ solely could provide a satisfactory level of regeneration (37.5–50%), while BA could not induce a shoot or calli without the presence of auxin. Contrary to our results, Skała and Wysokińska (2004) reported that 4.4 μM BA alone provoked direct regeneration from leaf explants of *S. nemorosa*. This difference may be due to diversity in the genotypes studied in the two types of research. In fact, diverse genotypes of one species may exhibit different *in-vitro* responses (Khaliluev et al., 2014; Sabbadini et al., 2019). Thidiazuron is a synthetic plant growth regulator, and it has a potent functionality for morphogenic processes such as shoot organogenesis and proliferation, callus induction, and somatic embryogenesis in tissue culture (Dewir et al., 2018). In the previous report, the shoot induction of Woodland Sage was achieved by BA as cytokinin and IAA and IBA as auxin (Skała and Wysokińska, 2004).

Our research exhibited that TDZ acted better than BA in high frequency direct and indirect organogenesis in Woodland Sage. Moreover, in this regard, NAA was the better auxin. Some reports suggested that simultaneous use of TDZ and NAA in a culture medium with optimal level enhanced shoot regeneration from leaf explants (Guo et al., 2011). TDZ induces more adventitious shoots than other cytokinins for most of the species, especially in the Lamiaceae family, such as *Salvia x jamensis* (Fraternale et al., 2013), *Salvia miltiorrhiza* (Tsai et al., 2015), *Ajuga bracteosa* (Ali et al., 2018), *Scutellaria bornmuelleri* (Gharari et al., 2019).

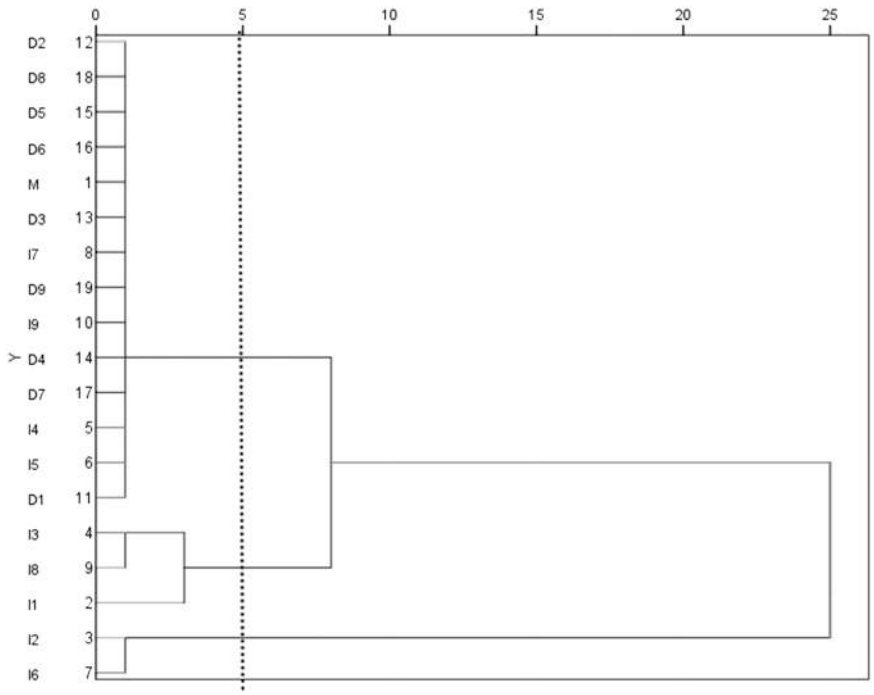

Fig. 6. - Dendrogram of cluster analysis of *S. nemorosa* based on rosmarinic acid content.

TDZ has both auxin and cytokinin activity, and its function relies on concentration and concomitant PGR (Guo et al., 2011). Our findings revealed that TDZ combined with 2, 4-D produced calli and, in combination with NAA, caused shoot regeneration.

According to our findings, 2, 4-D, among all the auxins used in the present experiment, had the best effect on *S. nemorosa* callus formation. As 2, 4-D concentration was raised in the medium, the amount of Woodland Sage callogenesis increased. Also, the presence of a low cytokinin level promoted this performance. In most reports published in callogenesis of *Salvia* genus such as *S. santolinifolia* (Jan et al., 2015, 2018), *S. officinalis* (Lemraski et al., 2014), *S. tomentosa* (Georgiev et al., 2014) and *S. splendens* (Huii et al., 2012), NAA was preferred as auxin source, and it gave better responses. However, Modarres et al. (2018) reported that high levels of 2, 4-D (3 mg/l) along with low levels of Kinetin (1 mg/l) were needed for callus induction in *S. leriifolia,* and it could not respond solely. However, NAA provided a more favorable response as 2, 4-D. These reports contradict our results.

Cytokinins are one of the principal components of the tissue culture medium for shoot multiplication. A proper cytokinin in an optimum concentration appears crucial to this process (Phillips and Garda, 2019). In the present study, attempts were performed to optimize the cytokinin concentration and type to gain healthy multiple shoots with robust growth in *S. nemorosa*. In the earlier report on Woodland Sage published by Skała and Wysokińska (2004), the maximum multiplication rate (5.7 shoots per explant) was achieved in 8.9 μM BA, and all levels of BA produce more shoots than TDZ. Despise it, in our study, TDZ, compared to BA, produced the highest shoots at all concentrations. Similar to our results reported on *Salvia x jamensis* (Fraternale et al., 2013). Also, Sharma et al. (2014) evaluated four cytokinin types (BA, TDZ, Kin, and 2iP) on shoot proliferation of *S. splendens* and stated that BA provided the highest multiplied shoots and shoot length compared to others. The consequence of BA than TDZ in shoot proliferation was reported on *S. fruticosa* (Arikat et al., 2004), *S. brachyodon* (Misic et al., 2006), *S. africana-lutea* (Makunga and van Staden, 2007) and *S. guaranitica* (Echeverrigaray et al., 2010) which is in contrast to our results. Overall, we found that adenine sulphate had a better cytokinin activity than the other cytokinins for *S. nemorosa* in vitro multiplication through nodal explants. Compared to TDZ and BA, AdS proliferated fewer shoots, but they were longer on average. On the other hand, shoots obtained by TDZ and BA exhibited a high level of hyperhydricity (28–54%). While shoots multiplied by AdS did not exhibit hyperhydric symptoms. Adenine sulphate may act synergistically as a cytokinin such as Kinetin or Zeatin, and it is a precursor for enhancing the biosynthesis of natural cytokinins. However, it is possible that AdS also prepares an additional nitrogen source for the cells, which can be taken up more rapidly than inorganic nitrogen. Thus, it can stimulate cell growth, somatic embryogenesis, and callogenesis and considerably enhance the shoot development in tissue culture (Mohd et al., 2014; Naaz et al., 2014). The promotive role of adenine sulphate in shoot proliferation has been described in different species, namely *Pithecellobium dulce* (Goyal et al., 2012), *Cichorium intybus* (Nandagopal and Ranjitha Kumari, 2006), *Althaea officinalis* (Naz and Anis, 2012), *Anacardium occidentale* (Martin, 2003) and *Stevia rebaudiana* (Mohd et al., 2014).

Hyperhydricity is a physiological and morphological abnormality observed in *in-vitro* propagated plants in response to culture conditions, basal medium components, mainly ammonium and plant growth regulator types, and concentrations, especially cytokinins (Liu et al., 2017). In our investigation, the only kind and concentration of cytokinins are diverse in different treatments, and hence, the observed hyperhydric plants were probably due to it. Hyperhydric shoots have higher endogenous cytokinin content than healthy shoots (Ivanova and Staden, 2010). Thidiazuron is more stable in plant tissues than other cytokinins because it is resistant to endogenous cytokinin oxidase. Furthermore, TDZ inhibits cytokinin oxidase activity, which causes an increase in purine cytokinins accumulation in plant tissues (Dewir et al., 2018). Thus, an increase in endogenous cytokinins may explain the high rate of hyperhydricity observed in multiplied shoots of *S. nemorosa* in a medium supplemented with TDZ. In *Pyrus pyrifolia*, Phenylurea derivatives cytokinins like TDZ increased approximately tenfold in the number of hyperhydric shoots than adenine derivatives (Kadota and Niimi, 2003). In several plant species such as *Dianthus caryophyllus* (Genkov et al., 1997), *Aloe polyphylla* (Ivanova and van Staden, 2008), *Cordyline fruticosa* (Dewir et al., 2015), and *Coleonema pulchellum* (Baskaran et al., 2014) TDZ-induced hyperhydricity of regenerated shoots was described.

Shoots incubated on PGRs free medium (control) could not develop adventitious roots at the end of the experiment. Therefore, the presence

of auxin in the rooting medium of the *S. nemorosa* seems necessary. Similar results were reported on other *Salvia* species, namely *S. splendens* (Yu et al., 2015), *S. miltiorrhiza* (Tsai et al., 2015), and *S. canariensis* (Mederos-Molina, 2004). Although, Skała and Wysokińska (2004) reported that *S. nemorosa* shoots could be produced 50% root on PGRs free medium, which does not coincide with our investigation. Our findings revealed that IAA was the better auxin than NAA and IBA in the rooting process because of providing an acceptable root number and length without forming a callus. Calli formation during rooting at the end of the shoot was not desirable because of its hard acclimatization (Sha Valli Khan et al., 1997; Veraplakorn, 2016).

The acclimatization protocol utilized for Woodland Sage in vitro raised plantlets was successful, and a 94% survival rate was attained. The present study exhibited a higher survival rate than other *Salvia* species, such as *S. nemorosa* (Skała and Wysokińska, 2004), *S. pratensis* (Ruffoni et al., 2007), *S. sclarea* (Grigoriadou et al., 2020).

To the best of our knowledge, this is the first report on evaluating somaclonal variation in the *in-vitro* culture of *Salvia* species. The ISSR outcomes were obtained from *in-vitro* derived plantlets, and the donor plant showed polymorphic bands. Plants regenerated through direct organogenesis were similar to source plants, and no polymorphic bands were seen. Vice versa, genetic instability has been detected in regenerants obtained by indirect organogenesis. Somaclonal variation is genetic or epigenetic changes found in the cell and tissue cultures, and this term is globally accepted for all forms of tissue culture-derived variants. Several factors, including the source of explants in-vitro propagation method, type and concentration of applied plant growth regulators, number and duration of subcultures, and stressful condition of *in-vitro* cultures, may cause somaclonal variation (Bairu et al., 2010; Miguel and Marum, 2011; Wang and Wang, 2012). In the present study, almost all culture conditions were the same, and the observed somaclonal variation was only between the two regeneration methods. Indirect organogenesis is provided by an intermediate callus phase, which is absent in direct organogenesis. Auxins applied during calli induction increased genetic variation by rising DNA-methylation rates (Bairu et al., 2010). In addition, they are often associated with genetic abnormalities such as polyploidy and endoreduplication due to DNA synthesis stimulation (Takagi et al., 2011). Also, 2, 4-D could cause genetic mutations during callus induction (Bouman and De Klerk, 2001). Thus, we can assume that maybe genetic changes during callus initiation are the main reason for the somaclonal variation observed in the indirect regeneration of Woodland Sage. For several plant species, such as *Titanotrichum oldhamii* (Takagi et al., 2011), strawberry (Biswas et al., 2009), *Chirita swinglei* (Chen et al., 2016), *Aloe vera* (Rathore et al., 2011) and *Bletilla striata* (Wang and Tian, 2014) somaclonal variation was observed in indirect regeneration. In comparison, direct regenerants showed genetic fidelity. Advantage of ISSR markers in the examination of genetic stability and somaclonal variation of *in-vitro* derived plantlets, similar to works on crops such as *Vanilla planifolia* (Pastelin Solano et al., 2019), *Puya berteroniana* (Viehmannova et al., 2016), *Withania coagulans* (Rathore et al., 2016), *Artemisia absinthium* (Kour et al., 2014) and *Moringa peregrine* (Al Khateeb et al., 2013) were validated by our findings.

In the current study, there was a significant difference in the activity of the PAL enzyme among indirect regenerants. In contrast, PAL activity was almost the same in direct regenerants. PAL is a branch point enzyme between the primary and the secondary metabolism, and is a key enzyme for regulating the influx of phenylalanine to the biosynthesis of phenolic compounds (Dong et al., 2010). Evidence has revealed that genetic, epigenetic changes, and external factors such as stress and elicitors can be affected PAL enzyme activity (Heydari et al., 2020b). In addition, several studies have confirmed that rosmarinic acid production directly correlates with PAL enzyme activity (Dong et al., 2010; Hou et al., 2020; Li et al., 2020). As a result, it can be concluded that the stability of PAL enzyme activity and the accumulation of rosmarinic acid among directly regenerated plants in the present study might explain the lack of genetic and epigenetic changes during direct organogenesis from *S. nemorosa* leaf explants. Also, according to the ISSR analysis results, the difference in the amount of rosmarinic acid production in indirect regenerants can be explained by genetic changes. Rosmarinic acid is a phenolic molecule with various applications, varying from food preservatives to cosmetics, and is also a molecule of interest to the pharmaceutical industry (Marchev et al., 2021). The RA possesses anti-inflammatory, anti-oxidative, antibacterial, antiviral, anti-hyperglycemic, anticancer, hepatoprotective, analgesic, cardioprotective, and neuroprotective properties. It is used in modern medicine to treat Alzheimer's disease, diabetes, fibrosis, and various types of cancer such as colorectal, breast, pancreatic, and lung cancers (Hitl et al., 2021; Trócsányi et al., 2020). Rosmarinic acid (RA) is the most abundant phenolic compound detected in Woodland Sage (Bahadori et al., 2017). Salvianolic acids, which have been used to treat cardiovascular disorders, fibrosis, and cancer, are another significant phenolic compound isolated from *S. nemorosa* (Heydari et al., 2020b).

5. Conclusion

The current study described an efficient and high-frequency *in vitro* regeneration protocol from leaf explants of *S. nemorosa* through direct and indirect organogenesis. The investigations revealed that the types, combinations, and ratios of plant growth regulators (PGRs) significantly affected direct organogenesis and callogenesis responses. TDZ, in combination with NAA, induced maximum direct adventitious shoot organogenesis from leaf explants, and by increasing the NAA ratio, the organogenesis rate decreased. The highest callus induction was observed when 2, 4-D was combined with BA or TDZ at a 4:1 ratio, and callogenesis decreased as this ratio declined. Our finding showed that TDZ produced maximum adventitious shoot during the proliferation stage, but most of them (40–50%) showed hyperhydric symptoms. Thus, adenine Sulphate is preferred as a PGR with cytokinin activity because it proliferated adventitious shoots without hyperhydricity. The plantlets rooted well in all the tested auxin. All the rooted plantlets were acclimatized with 94% survival in field conditions. For the first time, molecular and biochemical markers have been employed in present study to investigate somaclonal variations in *Salvia* species *in vitro* regeneration. ISSR and biochemical analysis confirmed the true-to-type nature of direct regenerated plantlets. Moreover, these analyses confirmed that somaclonal variation occurred during indirect organogenesis through the callus. The present direct organogenesis protocol is highly efficient and suited for multiplication and transgenic studies to improve the ornamental and medicinal traits of Woodland Sage. Furthermore, the indirect organogenesis protocol may promote the selection of somaclonal variants, which may help select ornamentally/ pharmaceutically important variants of commercial value.

CRediT authorship contribution statement

Hamid Reza Heydari: Data curation, Methodology, Software, Investigation, Writing - original draft, Writing - review & editing. **Esmaeil Chamani:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

References

Al Khateeb, W., Bahar, E., Lahham, J., Schroeder, D., Hussein, E., 2013. Regeneration and assessment of genetic fidelity of the endangered tree *Moringa peregrina* (Forsk.) fiori using inter simple sequence repeat (ISSR). Physiol. Mol. Biol. Plant.: Int. J. Funct. Plant. Biol. 19, 157–164. https://doi.org/10.1007/s12298-012-0149-z.

Aleksic, J.M., Stojanovic, D., Banovic, B., Jancic, R., 2012. A simple and efficient DNA isolation method for *Salvia officinalis*. Biochem Genet 50, 881–892. https://doi.org/10.1007/s10528-012-9528-y.

Ali, H., Khan, M.A., Kayani, W.K., Khan, T., Mashwani, Z.-u-R., Nazif, U., Khan, R.S., 2018. Thidiazuron regulated growth, secondary metabolism and essential oil profiles in shoot cultures of *Ajuga bracteosa*. Ind. Crops Prod. 121, 418–427. https://doi.org/10.1016/j.indcrop.2018.05.043.

Arikat, N.A., Jawad, F.M., Karam, N.S., Shibli, R.A., 2004. Micropropagation and accumulation of essential oils in wild sage (*Salvia fruticosa* Mill.). Sci. Hortic. 100, 193–202. https://doi.org/10.1016/j.scienta.2003.07.006.

Arora, K., Rai, M.K., Sharma, A.K., 2022. Tissue culture mediated biotechnological interventions in medicinal trees: recent progress. Plant Cell, Tissue Organ Cult. (PCTOC). https://doi.org/10.1007/s11240-022-02298-1.

Bahadori, M.B., Asghari, B., Dinparast, L., Zengin, G., Sarikurkcu, C., Abbas-Mohammadi, M., Bahadori, S., 2017. *Salvia nemorosa* L.: a novel source of bioactive agents with functional connections. LWT - Food Sci. Technol. 75, 42–50. https://doi.org/10.1016/j.lwt.2016.08.048.

Bairu, M.W., Aremu, A.O., Van Staden, J., 2010. Somaclonal variation in plants: causes and detection methods. Plant Growth Regul. 63, 147–173. https://doi.org/10.1007/s10725-010-9554-x.

Baskaran, P., Moyo, M., Van Staden, J., 2014. In vitro plant regeneration, phenolic compound production and pharmacological activities of *Coleonema pulchellum*. South Afr. J. Bot. 90, 74–79. https://doi.org/10.1016/j.sajb.2013.10.005.

Bhusare, B.P., John, C.K., Bhatt, V.P., Nikam, T.D., 2018. In vitro propagation of *Digitalis lanata* Ehrh. through direct shoot regeneration – A source of cardiotonic glycosides. Ind. Crops Prod. 121, 313–319. https://doi.org/10.1016/j.indcrop.2018.05.019.

Biswas, M.K., Dutt, M., Roy, U.K., Islam, R., Hossain, M., 2009. Development and evaluation of in vitro somaclonal variation in strawberry for improved horticultural traits. Sci. Hortic. 122, 409–416. https://doi.org/10.1016/j.scienta.2009.06.002.

Bouman, H., De Klerk, G.-J., 2001. Measurement of the extent of somaclonal variation in begonia plants regenerated under various conditions. Comp. three assays. Theor. Appl. Genet. 102, 111–117. https://doi.org/10.1007/s001220051625.

Chen, Y., Zhang, Y., Cheng, Q., Niu, M., Liang, H., Yan, H., Zhang, X., Teixeira da Silva, J. A., Ma, G., 2016. Plant regeneration via direct and callus-mediated organogenesis from leaf explants of *Chirita swinglei* (Merr.) W. T. Wang. Vitr. Cell. Dev. Biol. -Plant 52, 521–529. https://doi.org/10.1007/s11627-016-9766-5.

Dewir, Y., El-Mahrouk, M., El-Banna, A., 2015. In vitro propagation and preliminary results of Agrobacterium-mediated genetic transformation of *Cordyline fruticosa*. South Afr. J. Bot. 98, 45–51.

Dewir, Y.H., Nurmansyah, Naidoo, Y., Teixeira da Silva, J.A., 2018. Thidiazuron-induced abnormalities in plant tissue cultures. Plant Cell Rep. https://doi.org/10.1007/s00299-018-2326-1.

Dharman, A.K., Anilkumar, M., 2021. Micropropagation through indirect organogenesis in *Solanum capsicoides* All., and assessment of clonal fidelity using ISSR markers. Vitr. Cell. Dev. Biol. -Plant. https://doi.org/10.1007/s11627-020-10145-1.

Dong, J., Wan, G., Liang, Z., 2010. Accumulation of salicylic acid-induced phenolic compounds and raised activities of secondary metabolic and antioxidative enzymes in *Salvia miltiorrhiza* cell culture. J. Biotechnol. 148, 99–104. https://doi.org/10.1016/j.jbiotec.2010.05.009.

Echeverrigaray, S., Carrer, R.P., Andrade, L.B., 2010. Micropropagation of *Salvia guaranitica* Benth. through axillary shoot proliferation. Braz. Arch. Biol. Technol. 53, 883–888. https://doi.org/10.1590/S1516-89132010000400018.

Fraternale, D., Bisio, A., Ricci, D., 2013. Salvia x jamensis J. Compton: In vitro regeneration of shoots through TDZ and BA. In: Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology, 147, pp. 713–716. https://doi.org/10.1080/11263504.2013.788570.

Gao, H., Xu, P., Li, J., Ji, H., An, L., Xia, X., 2017. AgNO3 prevents the occurrence of hyperhydricity in *Dianthus chinensis* L. by enhancing water loss and antioxidant capacity. Vitr. Cell. Dev. Biol. -Plant 53, 561–570. https://doi.org/10.1007/s11627-017-9871-0.

Genkov, T., Tsoneva, P., Ivanova, I., 1997. Effect of cytokinins on photosynthetic pigments and chlorophyllase activity in *in vitro* cultures of axillary buds of *dianthus caryophyllus* L. J. Plant Growth Regul. 16, 169–172. https://doi.org/10.1007/pl00006992.

Georgiev, V., Marchev, A., Haas, C., Weber, J., Nikolova, M., Bley, T., Pavlov, A., 2014. Production of oleanolic and ursolic acids by callus cultures of *salvia tomentosa*Mill. Biotechnol. Biotechnol. Equip. 25, 34–38. https://doi.org/10.5504/bbeq.2011.0129.

Gharari, Z., Bagheri, K., Sharafi, A., Danafar, H., 2019. Thidiazuron induced efficient *in vitro* organogenesis and regeneration of *Scutellaria bornmuelleri*: an important medicinal plant. Vitr. Cell. Dev. Biol. -Plant 55, 133–138. https://doi.org/10.1007/s11627-019-09965-7.

Goyal, P., Kachhwaha, S., Kothari, S.L., 2012. Micropropagation of *Pithecellobium dulce* (Roxb.) Benth-a multipurpose leguminous tree and assessment of genetic fidelity of micropropagated plants using molecular markers. Physiol. Mol. Biol. Plant.: Int. J. Funct. Plant.Biol. 18, 169–176. https://doi.org/10.1007/s12298-012-0112-z.

Grigoriadou, K., Trikka, F.A., Tsoktouridis, G., Krigas, N., Sarropoulou, V., Papanastasi, K., Maloupa, E., Makris, A.M., 2020. Micropropagation and cultivation of *Salvia sclarea* for essential oil and sclareol production in northern Greece. Vitr. Cell. Dev. Biol. -Plant 56, 51–59. https://doi.org/10.1007/s11627-019-10040-4.

Guo, B., Bilal, H.A., Amir, Z., L, L.X., Y, H.W., 2011. Thidiazuron: a multi-dimensional plant growth regulator. Afr. J. Biotechnol. 10, 8984–9000. https://doi.org/10.5897/ajb11.636.

Heydari, H.R., Chamani, E., Esmaeilpour, B., 2020a. Effect of total nitrogen content and NH4+/NO3- ratio on biomass accumulation and secondary metabolite production in cell culture of *S. nemorosa*. Iran. J. Genet. Plant Breed. 9, 17–27. https://doi.org/10.30479/ijgpb.2020.12321.1258.

Heydari, H.R., Chamani, E., Esmaeilpour, B., 2020b. Cell line selection through gamma irradiation combined with multi-walled carbon nanotubes elicitation enhanced phenolic compounds accumulation in *Salvia nemorosa* cell culture. Plant Cell, Tissue Organ Cult. (PCTOC) 142, 353–367. https://doi.org/10.1007/s11240-020-01867-6.

Hitl, M., Kladar, N., Gavarić, N., Božin, B., 2021. Rosmarinic acid–human pharmacokinetics and health benefits. Planta Med. 87, 273–282.

Hou, Z., Li, Y., Su, F., Chen, J., Zhang, X., Xu, L., Yang, D., Liang, Z., 2020. Application of (1)H NMR combined with qRT-PCR technology in the exploration of rosmarinic acid biosynthesis in hair roots of *Salvia miltiorrhiza* Bunge and *Salvia castanea f. tomentosa* Stib. Planta 253, 2. https://doi.org/10.1007/s00425-020-03506-y.

Huii, L., Guoping, Z., Guozheng, S., Songlin, R., Qiaojuan, F., 2012. Callus Induction and Plant Regeneration from Mature Seeds of *Salvia splendens*. Int. J. Agric. Biol. 14.

Ivanova, M., Staden, J., 2010. Influence of gelling agent and cytokinins on the control of hyperhydricity in *Aloe polyphylla*. Plant Cell, Tissue Organ Cult. (PCTOC) 104, 13–21. https://doi.org/10.1007/s11240-010-9794-5.

Ivanova, M., van Staden, J., 2008. Effect of ammonium ions and cytokinins on hyperhydricity and multiplication rate of in vitro regenerated shoots of *Aloe polyphylla*. Plant Cell, Tissue Organ Cult. 92, 227–231. https://doi.org/10.1007/s11240-007-9311-7.

Jan, T., Naqvi, B., Qadri, R., 2015. In vitro mass multiplication and isolation of natural products from callus tissues of *Salvia santolinifolia* (Boiss). Bio Chem. Res 320–328.

Jan, T., Qadri, R., Naqvi, B., Adhikari, A., Nadeem, S., Muhammad, A., 2018. A novel Salvia lactomine from the callus culture of *Salvia santolinifolia* Boiss. Nat. Prod. Res. 32, 749–754. https://doi.org/10.1080/14786419.2017.1311887.

Kadota, M., Niimi, Y., 2003. Effects of cytokinin types and their concentrations on shoot proliferation and hyperhydricity in in vitro pear cultivar shoots. Plant Cell, Tissue Organ Cult. 72, 261–265. https://doi.org/10.1023/A:1022378511659.

Kaprinyak, T., Fari, M., 2019. Horticultural uses of botanical variations of woodland sage (*Salvia nemorosa* L.). Ecocycles 5, 24–32. https://doi.org/10.19040/ecocycles.v5i2.148.

Khaliluev, M.R., Bogoutdinova, L.R., Baranova, G.B., Baranova, E.N., Kharchenko, P.N., Dolgov, S.V., 2014. Influence of genotype, explant type, and component of culture medium on *in vitro* callus induction and shoot organogenesis of tomato (*Solanum lycopersicum* L.). Biol. Bull. 41, 512–521. https://doi.org/10.1134/s1062359014060041.

Kour, B., Kour, G., Kaul, S., Dhar, M.K., 2014. In vitro mass multiplication and assessment of genetic stability of *in vitro* raised *Artemisia absinthium* L. Plants Using ISSR and SSAP Molecular Markers. Adv. Bot. 2014, 7. https://doi.org/10.1155/2014/727020.

Krishna, H., Alizadeh, M., Singh, D., Singh, U., Chauhan, N., Eftekhari, M., Sadh, R.K., 2016. Somaclonal variations and their applications in horticultural crops improvement. 3 Biotech 6, 54. https://doi.org/10.1007/s13205-016-0389-7.

Kuźma, Ł., Wysokińska, H., 2003. Production of secondary metabolites in shoots of *Salvia nemorosa* L. cultured *in vitro*. Biotechnologia 63 (4), 154–159.

Lemraski, M.G., Eftekhari, M., Faraji, M., Zarrini, S.S., 2014. Study of callus induction in common sage (*Salvia officinalis* L.). Int. J. Agric. Crop Sci. 7, 386.

Li, J., Li, B., Luo, L., Cao, F., Yang, B., Gao, J., Yan, Y., Zhang, G., Peng, L., Hu, B., 2020. Increased phenolic acid and tanshinone production and transcriptional responses of biosynthetic genes in hairy root cultures of *Salvia przewalskii* Maxim. treated with methyl jasmonate and salicylic acid. Mol. Biol. Rep. https://doi.org/10.1007/s11033-020-05899-1.

Liu, M., Jiang, F., Kong, X., Tian, J., Wu, Z., Wu, Z., 2017. Effects of multiple factors on hyperhydricity of *Allium sativum* L. Sci. Hortic. 217, 285–296. https://doi.org/10.1016/j.scienta.2017.02.010.

Makunga, N.P., van Staden, J., 2007. An efficient system for the production of clonal plantlets of the medicinally important aromatic plant: *salvia africana-lutea* L. Plant Cell, Tissue Organ Cult. 92, 63–72. https://doi.org/10.1007/s11240-007-9305-5.

Marchev, A., Haas, C., Schulz, S., Georgiev, V., Steingroewer, J., Bley, T., Pavlov, A., 2014. Sage in vitro cultures: a promising tool for the production of bioactive terpenes and phenolic substances. Biotechnol. Lett. 36, 211–221. https://doi.org/10.1007/s10529-013-1350-z.

Marchev, A.S., Vasileva, L.V., Amirova, K.M., Savova, M.S., Koycheva, I.K., Balcheva-Sivenova, Z.P., Vasileva, S.M., Georgiev, M.I., 2021. Rosmarinic acid - From bench to valuable applications in food industry. Trends Food Sci. Technol. 117, 182–193. https://doi.org/10.1016/j.tifs.2021.03.015.

Martin, K.P., 2003. Plant regeneration through direct somatic embryogenesis on seed coat explants of cashew (Anacardium occidentale L.). Sci. Hortic. 98, 299–304. https://doi.org/10.1016/S0304-4238(03)00005-0.

Martínez-Estrada, E., Caamal-Velázquez, J.H., Salinas-Ruíz, J., Bello-Bello, J.J., 2017. Assessment of somaclonal variation during sugarcane micropropagation in temporary immersion bioreactors by intersimple sequence repeat (ISSR) markers. Vitr. Cell. Dev. Biol. -Plant 53, 553–560. https://doi.org/10.1007/s11627-017-9852-3.

Mederos-Molina, S., 2004. In vitro callus induction and plants from stem and petiole explants of *Salvia canariensis* L. Plant Tissue Cult. 14, 167–172.

Mederos-Molina, S., 2006. Micropropagation of *Salvia broussonetii* Benth.-a medicinal plant species. Plant Tissue Cult. Biotechnol. 16, 19–23.

Miguel, C., Marum, L., 2011. An epigenetic view of plant cells cultured in vitro: somaclonal variation and beyond. J. Exp. Bot. 62, 3713–3725. https://doi.org/10.1093/jxb/err155.

Mirza, M., Sefidkon, F., 1999. Essential oil composition of two Salvia species from Iran, *Salvia nemorosa* L. and *Salvia reuterana* Boiss. Flavour Fragr. J. 14, 230–232. https://doi.org/10.1002/(SICI)1099-1026(199907/08)14:4%3C230::AID-FFJ816%3E3.0.CO;2-L.

Misic, D., Grubisic, D., Konjevic, R., 2006. Micropropagation of *Salvia brachyodon* through nodal explants. Biol. Plant. 50, 473–476.

Modarres, M., Esmaeilzadeh Bahabadi, S., Taghavizadeh Yazdi, M.E., 2018. Enhanced production of phenolic acids in cell suspension culture of *Salvia leriifolia* Benth. using growth regulators and sucrose. Cytotechnology 70, 741–750. https://doi.org/10.1007/s10616-017-0178-0.

Mohd, K.K., Pragati, M., Taru, S., P, K.S., P, W.R., 2014. Effect of adenine sulphate on *in vitro* mass propagation of *Stevia rebaudiana* Bertoni. J. Med. Plants Res. 8, 543–549. https://doi.org/10.5897/jmpr2013.5217.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15, 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

Naaz, A., Shahzad, A., Anis, M., 2014. Effect of adenine sulphate interaction on growth and development of shoot regeneration and inhibition of shoot tip necrosis under in vitro condition in adult *Syzygium cumini* L.–a multipurpose tree. Appl. Biochem Biotechnol. 173, 90–102. https://doi.org/10.1007/s12010-014-0797-2.

Nandagopal, S., Ranjitha Kumari, B., 2006. Adenine sulphate induced high frequency shoot organogenesis in callus and *in vitro* flowering of *Cichorium intybus* L. cv. Focus-a potent medicinal plant. Acta Agric. Slov. 87, 415–425.

Nayak, S.A., Kumar, S., Satapathy, K., Moharana, A., Behera, B., Barik, D.P., Acharya, L., Mohapatra, P.K., Jena, P.K., Naik, S.K., 2012. In vitro plant regeneration from cotyledonary nodes of *Withania somnifera* (L.) Dunal and assessment of clonal fidelity using RAPD and ISSR markers. Acta Physiol. Plant. 35, 195–203. https://doi.org/10.1007/s11738-012-1063-2.

Naz, R., Anis, M., 2012. Acceleration of adventitious shoots by interaction between exogenous hormone and adenine sulphate in *Althaea officinalis* L. Appl. Biochem Biotechnol. 168, 1239–1255. https://doi.org/10.1007/s12010-012-9853-y.

Ngezahayo, F., 2018. Somaclonal Variations and Their Applications in Medicinal Plant Improvement. Biotechnological Approaches for Medicinal and Aromatic Plants. Springer,, pp. 503–519.

Niazian, M., 2019. Application of genetics and biotechnology for improving medicinal plants. Planta 249, 953–973. https://doi.org/10.1007/s00425-019-03099-1.

Nikolova, M., Aneva, I., 2017. European Species of Genus Salvia: Distribution, Chemodiversity and Biological Activity. In: Georgiev, V., Pavlov, A. (Eds.), Salvia Biotechnology. Springer International Publishing, Cham, pp. 1–30.

Pastelin Solano, M.C., Salinas Ruiz, J., Gonzalez Arnao, M.T., Castaneda Castro, O., Galindo Tovar, M.E., 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). Physiol. Mol. Biol. Plant.: Int. J. Funct. Plant.Biol. 25, 561–567. https://doi.org/10.1007/s12298-019-00645-9.

Pawełkowicz, M.E., Skarzyńska, A., Mróz, T., Bystrzycki, E., Pląder, W., 2021. Molecular insight into somaclonal variation phenomena from transcriptome profiling of cucumber (*Cucumis sativus* L.) lines. Plant Cell, Tissue Organ Cult. (PCTOC). https://doi.org/10.1007/s11240-020-02005-y.

Peng, X., Zhang, T.-t, Zhang, J., 2015. Effect of subculture times on genetic fidelity, endogenous hormone level and pharmaceutical potential of *Tetrastigma hemsleyanum* callus. Plant Cell, Tissue Organ Cult. (PCTOC) 122, 67–77. https://doi.org/10.1007/s11240-015-0750-2.

Phillips, G.C., Garda, M., 2019. Plant tissue culture media and practices: an overview. Vitr. Cell. Dev. Biol. -Plant 55, 242–257. https://doi.org/10.1007/s11627-019-09983-5.

Rathore, M.S., Chikara, J., Mastan, S.G., Rahman, H., Anand, K.G., Shekhawat, N.S., 2011. Assessment of genetic stability and instability of tissue culture-propagated plantlets of *Aloe vera* L. by RAPD and ISSR markers. Appl. Biochem Biotechnol. 165, 1356–1365. https://doi.org/10.1007/s12010-011-9352-6.

Rathore, M.S., Mastan, S.G., Yadav, P., Bhatt, V.D., Shekhawat, N.S., Chikara, J., 2016. Shoot regeneration from leaf explants of *Withania coagulans* (Stocks) Dunal and genetic stability evaluation of regenerates with RAPD and ISSR markers. South Afr. J. Bot. 102, 12–17. https://doi.org/10.1016/j.sajb.2015.08.003.

Ruffoni, B., Savona, M., Capponi, A., Campagna, G., Cervelli, C., 2007. Micropropagation of *Salvia pratensis* L. and *Salvia nemorosa* L. accessions selected for ornamental characters. III Int. Symp. . Acclim. Establ. Micro Plants 812, 201–204.

Sabbadini, S., Capriotti, L., Molesini, B., Pandolfini, T., Navacchi, O., Limera, C., Ricci, A., Mezzetti, B., 2019. Comparison of regeneration capacity and Agrobacterium-mediated cell transformation efficiency of different cultivars and rootstocks of *Vitis* spp. via organogenesis. Sci. Rep. 9, 582. https://doi.org/10.1038/s41598-018-37335-7.

Sha Valli Khan, P.S., Prakash, E., Rao, K.R., 1997. In vitro micropropagation of an endemic fruit tree *Syzygium alternifolium* (Wight) walp. Plant Cell Rep. 16, 325–328. https://doi.org/10.1007/BF01088290.

Sharma, S., Shahzad, A., Kumar, J., Anis, M., 2014. In vitro propagation and synseed production of scarlet salvia (*Salvia splendens*). Rend. Lince-.-. 25, 359–368. https://doi.org/10.1007/s12210-014-0308-y.

Skała, E., Wysokińska, H., 2004. In vitro regeneration of *Salvia nemorosa* L. from shoot tips and leaf explants. Vitr. Cell. Dev. Biol. -Plant 40, 596–602. https://doi.org/10.1079/ivp2004580.

Takagi, H., Sugawara, S., Saito, T., Tasaki, H., Yuanxue, L., Kaiyun, G., Han, D.-S., Godo, T., Nakano, M., 2011. Plant regeneration via direct and indirect adventitious shoot formation and chromosome-doubled somaclonal variation in *Titanotrichum oldhamii* (Hemsl.) Solereder. Plant Biotechnol. Rep. 5, 187–195. https://doi.org/10.1007/s11816-011-0172-5.

Thakur, M., Sharma, V., Luharch, R., 2021. Propagation of plum (*Prunus salicina* L.) cultivar Frontier in vitro through control of shoot tip necrosis (STN) and validation of genetic integrity using ISSR markers. Plant Physiol. Rep. https://doi.org/10.1007/s40502-021-00580-6.

Trócsányi, E., György, Z., Zámboriné-Németh, É., 2020. New insights into rosmarinic acid biosynthesis based on molecular studies. Curr. Plant Biol. https://doi.org/10.1016/j.cpb.2020.100162.

Tsai, K.-L., Chen, E.G., Chen, J.-T., 2015. Thidiazuron-induced efficient propagation of *Salvia miltiorrhiza* through in vitro organogenesis and medicinal constituents of regenerated plants. Acta Physiol. Plant. 38 https://doi.org/10.1007/s11738-015-2051-0.

Ulvrova, T., Vitamvas, J., Cepkova, P.H., Eliasova, K., Janovska, D., Bazant, V., Viehmannova, I., 2021. Micropropagation of an ornamental shrub *Disanthus cercidifolius* Maxim. and assessment of genetic fidelity of regenerants using ISSR and flow cytometry. Plant Cell, Tissue Organ Cult. (PCTOC) 144, 555–566. https://doi.org/10.1007/s11240-020-01978-0.

Veraplakorn, V., 2016. Micropropagation and callus induction of *Lantana camara* L. – A medicinal plant. Agric. Nat. Resour. 50, 338–344. https://doi.org/10.1016/j.anres.2016.12.002.

Viehmannova, I., Cepkova, P.H., Vitamvas, J., Streblova, P., Kisilova, J., 2016. Micropropagation of a giant ornamental bromeliad *Puya berteroniana* through adventitious shoots and assessment of their genetic stability through ISSR primers and flow cytometry. Plant Cell, Tissue Organ Cult. (PCTOC) 125, 293–302. https://doi.org/10.1007/s11240-016-0949-x.

Wang, C.X., Tian, M., 2014. Callus-mediated and direct protocorm-like body formation of *Bletilla striata* and assessment of clonal fidelity using ISSR markers. Acta Physiol. Plant. 36, 2321–2330. https://doi.org/10.1007/s11738-014-1594-9.

Wang, Q.-M., Wang, L., 2012. An evolutionary view of plant tissue culture: somaclonal variation and selection. Plant Cell Rep. 31, 1535–1547. https://doi.org/10.1007/s00299-012-1281-5.

Wu, C.F., Karioti, A., Rohr, D., Bilia, A.R., Efferth, T., 2016. Production of rosmarinic acid and salvianolic acid B from callus culture of *Salvia miltiorrhiza* with cytotoxicity towards acute lymphoblastic leukemia cells. Food Chem. 201, 292–297. https://doi.org/10.1016/j.foodchem.2016.01.054.

Yu, L., Chen, H., Hong, P., Wang, H., Liu, K., 2015. Adventitious bud induction and plant regeneration from stem nodes of *salvia splendens* 'Cailinghong'. HortScience 50, 869–872. https://doi.org/10.21273/HORTSCI.50.6.869.