ORIGINAL ARTICLE

RECORDS OF PHARMACEUTICAL AND BIOMEDICAL SCIENCES

In Vitro Propagation and Conservation of *Solenostemma argel* (Delile), an Endangered Medicinal Plant in Sinai Peninsula

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Received: 15. 04. 2025

Revised: 25. 06. 2025

Accepted: 28. 06. 2025

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Abstract

Solenostemma argel (*S. argel*) is an endangered medicinal plant in the Sinai Peninsula with numerous pharmaceutical applications. The propagation of this plant through biotechnological tissue culture approaches is crucial due to the increasing demand in various industries. This research sought to investigate the *in vitro* propagation of *S. argel* using seed germination, determine the best culture medium, and assess the genetic similarity of regenerated plants by the ISSR approach. Seeds were collected, and *in vitro* seedlings were used for shoot induction. Shoot tips were cultured on MS media with 1, 2, 4, and 8 mg/L of cytokinins such as benzyl adenine, 2-isopentenyladenine, kinetin, and zeatin. Additionally, the MS medium for root induction was enriched with 1, 2, 4, and 8 mg/L of each auxin IAA, IBA, and NAA. Low levels of zeatin (1 or 2 mg/L) and kinetin (up to 8 mg/L) added to the MS medium were most effective in promoting increased growth, resulting in higher numbers of branches, leaves, and longer and heavier weights of cultured *S. argel*. The best results for rooting percentage, number of adventitious roots, their length, and fresh weight were shown in MS medium supplemented with 4 mg/L of IBA. ISSR markers proved the genetic stability and uniformity of regenerated plants. Collectively, the efficiency of micropropagation and rooting can be significantly improved by incorporating plant growth regulators such as zeatin or kinetin, along with auxins like IBA. This study could facilitate the exploitation of *S. argel* by the pharmaceutical industry and contribute to conservation strategies.

Keywords: *Solenostemma argel*, Endangered medicinal plant, *In vitro* propagation.

1. Introduction

Medicinal herbs are used as an ancient form of medication as far back as 60 000 years ago in western and eastern cultures (**Gossell-Williams *et al.*, 2006**). Sinai is regarded as an expansive nature reserve, sustaining plants, animals, and marine organisms with historic habitats that date back thousands of years. Numerous species of Sinai Peninsula's flora have been conventionally employed by the local Bedouin societies because they are essential sources of pharmaceuticals that are effective in ailment treatment and sustainable human health (**Dasgupta, 2023; El-Khalafy *et al.*, 2023**).

Solenostemma argel (Delile) Hayne (Family: Asclepiadaceae) is an Egyptian wild perennial desert shrub, whose local Arabic call is Hargal or Argel. It is used by local Bedouin populations as a remedy for colon disorders, jaundice and cystitis, neuralgia, abdominal cramps, inflammation, rheumatism, spasm, and diabetes (**Ibrahim *et al.*, 2015**). Of late, the ethanolic extract of *S. argel* was demonstrated to have a strong repressive action counter to pancreatic

lipase enzyme (**Benmaarouf *et al.*, 2020**), as well as exhibited several biological activities such as hypoglycemic (**Al-Deen and Al-Naqeb, 2014**), hepatoprotective (**D Azer *et al.*, 2021**), anti-Alzheimer (**Demmak *et al.*, 2019**), anti-obesity (**El-Shiekh *et al.*, 2019**), gastroprotective effect (**El-Shiekh *et al.*, 2021a**), anti-rheumatoid arthritis (**El-Shiekh *et al.*, 2021b**) and hypolipidemic effects (**Dasgupta, 2023**). Prior biochemical research in *S. argel* confirmed the existence of flavonoids (main active metabolites), phenolic acids, triterpenes, monoterpenes glycosides, pregnane, pregnane glycosides, and acylated phenolic glycosides (**El-Shiekh *et al.*, 2021b**). Owing to overexploitation of *S. argel* (Del.) for several medicinal purposes, it is recently considered as a vulnerable endangered plant (**Amar, 2010; Moustafa and Mansour, 2020**). *S. argel* (Del.) faces a risk of multiple threats, including climate changes, decline in pollinators' population, habitat destruction, or disruption of beneficial mycorrhizal associations, and poor harvesting practices (**Omar and Elgamal, 2021**).

To overcome these limitations, this study aimed to establish a practical *in vitro* micropropagation protocol for the sustainable conservation of Egyptian *S. argel* (Del.). Additionally, after the acclimation period, we assessed the genetic similarity and stability of regenerated and also the adapted plants.

2. Material and Methods

2.1. Collection of plants:

In August 2022, the aerial part of *S. argel* (Del.) plant was collected from its habitat in the Wadi Zaghra area, South Sinai, Egypt (GPS: 28° 39' 04.0" N, 34° 20' 47.9" E). The plant was identified by a plant taxonomist Dr. Samir S. Telep, Botany Department, Faculty of Science, Zagazig University, Zagazig, Egypt.

2.2. *In vitro* micro propagation protocol:

2.2.1. Seed sterilization and mother stock establishment

The culture medium was prepared as follows: 0.22 % (w/v) Murashige and Skoog medium (MS, Phyto Technology Laboratories®, US), 3% (w/v) sucrose and 0.6% (w/v) agar (PTC agar, Sigma, USA) were dissolved in 1000 mL of distilled water. The pH was adjusted to 5.7 ± 0.1 by adding 0.1 M HCl or 0.1 M NaOH as needed. The prepared medium was transferred into 50 mL culture tube, sterilized in the autoclave at 121°C for 20 minutes, and allowed to solidify (**Beyl, 2018**).

For seed sterilization, the seeds were removed from the fruits, soaked in a 70% ethanol solution for 30 seconds, rinsed with a 10 % sodium hypochlorite solution for 15 minutes and then washed three times with sterile tap water under laminar airflow (**da Silva *et al.*, 2015**). One hundred seeds were cultured with three seeds per 50 mL culture tube. The cultures were sustained in growth room environments at 25 ± 2 °C and 16 hours' photoperiods provided by a white LED tube with a light intensity of 100 µmol/m^2/s. The *in vitro* germinated plantlets were used for further propagation experiments.

2.2.2. *In vitro* shoot propagation:

Six-week-old *in vitro* seedlings were applied for shoot induction. The cotyledon, hypocotyl and roots were carefully removed, and then about 0.5-1 cm shoot tip were cultured on full strength MS medium (0.44 % w/v) supplemented with sucrose (3% w/v), agar (0.6% w/v), and diverse concentrations of cytokinins, benzyl adenine (BA), 2-isopentenyladenine (2iP), kinetin (Kin.), and zeatin, at concentration 1, 2, 4, and 8 mg/L. The BA, and Kin were purchased from Bio Basic Company (INC®, New York, USA), 2iP was acquired from Phyto Technology Laboratories® (Lenexa, USA), while the zeatin was acquired from Popatwadi (Mumbai 400002, India).

Each treatment consisted of 6 replicates and each replicate contained two micro shoots/replicates. The cultures were preserved under the same growth room conditions as mentioned above. Data regarding the number of new shoots per initial microshoot, fresh weight of plantlets, and maximum shoot height was collected and recorded during the experimental study.

2.2.3. *In vitro* adventitious root formation and acclimatization:

Six-week-old *in vitro* seedlings were used for shoot induction. The cotyledon, hypocotyl and roots were carefully removed, and then approximately 0.5-1 cm shoot tip were cultured on full strength MS medium (0.44 % w/v) supplemented with sucrose (3% w/v), agar (0.6% w/v), and different levels of auxins including; indole-3-acetic acid (IAA, Oxford®, Mumbai), 1-naphthalene acetic acid (NAA, Bio Basic INC®, Canada), or indole-3-butyric acid (IBA, Bio Basic INC®, Canada) at concentration 1, 2, 4, and 8 mg/L. Each treatment consisted of 6 replicates with each replicate containing two micro shoots. The cultures were preserved under the same growth room conditions as mentioned above. Data were collected on the number of roots per shoot tip, length of the longest roots, and rooting percentages. To acclimate the cultivated *S. argel* (Del.), the roots washed carefully with distilled water to reduce any remnants of gel media and prevent potential plant diseases. Plantlets are then transported to small pots filled with sterile soil. The plantlets are placed in shade for 5 days, receiving diffused natural light. Finally, the

plantlets are moved to larger pots with a mixture of sterile soil and sand and placed in indirect sunlight (**De Stefano *et al.*, 2022**).

2.2.4. Callus formation:

Leaf and root fragments were applied as explants for callus induction. The full-strength MS medium was supplemented with 2 mg of NAA and 1 mg of 2,4-dichlorophenoxyacetic acid (2,4-D, Bio Basic INC®, Canada) (**Fitriandi *et al.*, 2023**). Each treatment comprised 6 replicates, with each replicate containing three leaf or root explants. The cultures were kept under the same growth room environments as illustrated earlier. Data on callus characteristics such as colour, fresh weight, and dry weight were recorded.

2.3. ISSR-PCR analysis of wild and acclimated plant:

2.3.1. DNA Extraction

Young leaves (~400 mg) from five *S. argel* (Del.) cultivars were collected and exposed to genomic DNA isolation using the Plant DNeasy Mini Kit (Qiagen, Santa Clarita, CA) following the manufacturer's protocol. The DNA extracts underwent RNase-A treatment (100 mg/mL, Sigma, USA) for 30 minutes at 37°C (**Al-Khayri *et al.*, 2023**). The concentration of the extracted DNA was considered using a NanoDrop 2000 (Thermo Scientific™, Waltham, MA, USA) and accommodated to 50 ng/µL with Tris-EDTA (TE) buffer before being used for PCR amplifications (**Abdelghaffar *et al.*, 2023a**).

2.3.2. PCR Amplification

PCR amplification was carried out on the *S. argel* (Del.) genomic DNA using eight ISSR primers (Table 1). The reactions were conducted in a thermal cycler (Veriti™ 96-Well Fast) with a 25 µL reaction mixture comprising 3 µL of genomic DNA (50 ng/µL), 2.5 µl of 10x reaction buffer, 1.5 µl of each primer (5 µM), 2 units of Taq DNA polymerase (Promega) (5 U/µL), 5 mM $MgCl_2$ and 0.5 mM dNTPs. The amplification program included an initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 30 s for denaturation, 30 s at the annealing temperature, and 2 min at 72°C for extension, with a final extension of 5 minutes at the same temperature (**Al-Khayri, *et al.*, 2023; Abdelghaffar *et al.*, 2023b**).

2.3.3. Gel Electrophoresis:

The PCR products were separated into a 1.5% agarose gel with ethidium bromide staining at 90 volts for 30 minutes (**Abdelghaffar *et al.*, 2023a**). A 100 bp DNA ladder was used as a size standard. The amplicons were visualized using a Gel Doc EZ Imager from BIO-RAD.

2.4. Statistical analysis

The data were confirmed for homogeneity and normality by Levene's test and Shapiro-Wilk test, respectively. The data was analyzed using SPSS 26.One-way analysis of variance and Duncan's multiple comparison tests were used to indicate the statistical differences between all variables evaluated in this experiment. The values were considered statistically significant when the *P*-value was less than 0.05. Means and standard error of the mean (SEM) were documented to demonstrate the data.

3. Results and Discussion.

3.1. Seed germination and mother stock establishment:

The sterilization process was effective (Fig. 1) and the percentage of seed germination was 55.6818%, 68.1818% and 71.5909% after 8, 10 and 20 days, respectively. The highest germination percentage was determined to be 73.86% after 23 days from seed culture and then followed by a plateau phase.

3.2. *In vitro* shoot propagation

We conducted a study to measure the impact of growth regulators on shoot propagation in order to identify the most effective cytokinins for enhancing shoot propagation. The number of shoots, number of leaves, and their weights were recorded after supplementing MS medium with different levels of BA, 2iP, Zeatin, and Kin in the sixth week (Table 2).

The maximum number of shoots (5.83 ± 0.25) was noticed in MS medium enriched with 1 mg/L of zeatin (P <0.001; Figure 2A), while the lowest values of the number of shoots were recorded in MS medium supplemented with 2 mg/L of 2iP (1.33 ± 0.21) compared to the control group (1.50 ± 0.22; P <0.001). All media enriched with zeatin had a greater number of shoots compared to other groups. The higher concentration of zeatin, the smaller number of *in vitro* propagated shoots.

The number of leaves (29.66 ±0.49) was higher in MS medium supplemented with 1 mg/L of zeatin, followed by 2 mg/L (29.00 ± 0.73) and 4 mg/L (27.50±0.88) of Kin. The lowest number of leaves was observed 8 mg/L of BA (10.33±0.49; P < 0.001) compared to the control group (10.83±0.6).

The shoot length was higher in plants generated from the medium enriched with 4 mg/L of Kin with no significant differences compared to the 1 mg/L of zeatin group and 1 or 2 mg/L of Kin groups. The addition of BA or 2iP (8 mg/L) to the MS medium resulted in the shortest shoot length. The addition of Kin (1-8 mg/L) significantly increased the shoot length compared to other groups (P < 0.001).

So, lower levels of zeatin (1 or 2 mg/L) and Kin (up to 8 mg/L) were found to be most effective in promoting growth, resulting in maximum numbers of shoots, leaves, and increased length and weight.

The positive effects of Zeatin on *in vitro* seedling growth have been supported by many researchers. Cytokinin plays a crucial role as the primary plant hormone in stimulating cell division, differentiation, and overall plant growth and development. Its physiological function involves triggering RNA and protein synthesis, as well as enhancing enzyme activity. Various forms of cytokinin can be employed to generate multiple shoots by disrupting the dominance of the apical shoot. Only one study has been reported for *in vitro* propagation of this plant (**Abd Alhady *et al.*, 2016**). This study reported that adding BA (3 mg/L) or 2iP (1 mg/L) heightened the number of shoots/plants. However, in the current experiment, Kin or Zeatin exhibited better results for the number of branches, leaves, their length, and weights than those in BA and 2iP. Zeatin is key as a plant hormone to confirm an appropriate rate of proliferation and growth (**Nikolić *et al.*, 2006; Masekesa *et al.*, 2016**). The existing results are in agreement with those of **Marbawi *et al.* (2018**), who reported that 20 µM zeatin is the ideal concentration of plant growth regulator for shoot proliferation from nodal segments. Moreover, **Najhah *et al.* (2021)** clarified that 1–7 mg/L of zeatin produced up to 100% shoot regeneration in *Labisia pumila varieties*. In agreement with these findings, **Tekdal and Çetiner (2014)** demonstrated that zeatin was the most potent cytokinin for inducing shoot regeneration in *Thermopsis turcica*. Additionally, zeatin has been shown to promote shoot development (**Wang *et al.*, 2019**) in *Vaccinium corymbosum* L. and *Solanum nigrum* L. The enhanced efficacy of zeatin in inducing shoot regeneration can be accredited by its reduced affinity for cytokinin-degrading enzymes. As a result, zeatin is less prone to degradation, leading to higher intracellular concentrations (**Nikolić *et al.*, 2006**). This elevated concentration of zeatin creates a more conducive environment for organogenesis compared to other cytokinins at equivalent concentrations (**Masekesa *et al.*, 2016**). These results corroborate previous findings that zeatin is the most effective cytokinin for inducing shoot regeneration from cucumber nodal explants (**Abu-Romman *et al.*, 2015**). Zeatin treatment consistently yielded the highest percentage of regenerating explants, the greatest number of shoots per explant, and the longest shoot lengths (**Abu-Romman *et al.*, 2015**).

3.3. *In vitro* adventitious root formation and acclimatization:

We performed a study to compare the impacts of different levels of auxins on *in vitro* adventitious root formation in order to determine the most effective auxin for promoting root growth. The impact of various concentrations of IAA, IBA, and NAA on the number of roots, root length, explant fresh weight, and rooting percentage of *S. argel* is summarized in Table 3.

The addition of 4 mg/L of IBA to the MS medium substantially improved the number of roots in the explants compared to other treatments ($P < 0.001$; Figure 2B). The IAA at 1 mg /L had the lowest number of roots (0.83 ± 0.30) compared to the control group (0.33 ± 0.21).

All concentrations of IBA (except 8 mg/L) resulted in longer lengths of the longest roots compared to other groups ($P < 0.001$). The higher the concentration of the IBA, the shorter root length.

IBA at 4 mg/L significantly produced a higher percentage of rooting (65.0 ± 5.00) compared to other groups ($P < 0.001$).

Root formation is a significant challenge in the *in vitro* propagation of many herbal plants. Rooting of plants is typically stimulated by auxin administration. In this study, three auxins (NAA, IAA, and IBA) were assessed for root development due to high stability in plant *in vitro* culture. Our results revealed that IBA (4 mg/L) was found to be more stable than the other auxins NAA and IAA, making it the most extensively applied auxin for root induction in this endangered plant (**Wang *et al.*, 2019**).

The optimal medium for rooting axillary shoots of *S. argel* was half-strength MS medium extended with 4 mg/L IBA, resulting in 65% rooting with the highest mean length of axillary shoots, and mean number and length of roots per explant. These results are consistent with the findings of **Nitnaware *et al.* (2011)** for *Phyllanthus amarus* plantlets rooted in ½ MS + 0.5 mg/L of IBA after 3–4 weeks. A study by **Shiragave (2015),** stated the efficacy of IBA over other auxins in rooting *Phyllanthus reticulatus*. Additionally, *Paulownia tomentosa* and *Paulownia fortuneii* displayed the longest root lengths and highest root densities when cultivated on MS medium supplemented with 0.5 or 1.5 mg/L NAA (**Saiju *et al.*, 2018**). A higher root number is crucial for successful acclimatization and is indicative of a robust rooting response (**Raveendar *et al.*, 2019**).

In this study, approximately 70% of the *in vitro*-grown plants were successfully acclimatized to greenhouse conditions (Figure 2D). The successful transition of plantlets from the controlled *in vitro* environment to natural conditions is a critical step in any micropropagation system. *In vitro* culture can induce epigenetic variations in plant physiology. The transition to greenhouse and field environments,

characterized by lower humidity, higher light intensity, and increased pathogen pressure, can be stressful for *in vitro*-grown plants. Additionally, the unique conditions of *in vitro* culture can lead to abnormal morphological, anatomical, and physiological traits in plantlets.

3.4. Callus formation:

Optimizing culture media for callus formation and tissue regeneration in endangered plants, such as *S. argel*, has significant biotechnological implications. The resulting callus was a compact, nodular mass with a greenish-yellow colour, as depicted in Figure 2C and Table 4. The fresh and dry weights were significantly higher (P <0.001) in MS supplemented with NAA (2 mg/L) + 2,4-D (1 mg/L) compared to other groups (Table 4). Consistent with our observations, the incorporation of NAA (5.37 mg/L) and 2,4-D (2.26 mg/L) significantly augmented callus formation percentages from leaf of *Citrus grandis* (L.) Osbeck (pummelo), achieving 95.3±2.4% (**Tao *et al.*, 2022**). The enhanced efficacy attributable to the combination of NAA and 2,4-D for callus induction is most probable a consequence of their synergistic action in assisting vigorous cell proliferation in addition to the inhibitory effect of 2,4-D on root formation induced by NAA.

3.5. Molecular Profiling using ISSR technique for Genetic diversity analysis:

Genetic divergence analysis was conducted on two different genotypes of *S. argel* (mother plant and acclimatized plant) using eight ISSR primers, as shown in Table 5 and Figure 3. This method facilitated the identification of molecular polymorphisms between the two varieties. The eight primers generated consistent banding profiles that exhibited polymorphisms among the studied varieties, as depicted in Table 5 and Figure 3. The PCR reactions resulted in the detection of 23 polymorphic loci and 16 monomorphic loci. The level of polymorphism revealed by ISSR-PCR ranged from 40% with primer UBC826 to 100% with primers UBC825 and UCB811, with an average of 69.37% per primer. PCR analysis *of S. argel* cultivars was carried out using 3' anchored ISSR primers. Two *S. argel* cultivars were selected and screened using eight ISSR primers. The lowest polymorphism was observed in ISSR6, as indicated in Table 5. ISSR-PCR results (ISSR5 and ISSR7) for two different genotypes of *S. argel* showed an equal polymorphism value of 50%.

We also assessed the similarity coefficient and genetic distance to understand the differences between the mother plant, and adapted *S. argel* plant. In our results (Table 6), the adapted plant is similar to wild plant by (0.92) similarity coefficient. The clustering pattern of the *S. argel* was generated using bioinformatics software NTSYS pc 2.02 based on pairwise comparisons. Cluster analysis revealed genetic relationships with similar index values ranging from 0.5 to 3.5, with an average of 3.00.

Genetic changes can be induced by various factors during the *in vitro* regeneration of plants, making it crucial to evaluate how these factors affect DNA fingerprinting among all cultured plants and compare them with wild plants (**Karwasra *et al.*, 2024**). This report presents the first comprehensive study on the *in-vitro* growth of *S. argel*, assessing genetic stability through DNA fingerprinting using ISSR markers. Plant tissue culture undergoes high levels of oxidative stress, leading to DNA damage.

The genetic stability of *in-vitro*-grown plants was confirmed by ISSR primers, showing a close genetic resemblance to the mother plant. The ISSR-based PCR reactions resulted in bands scored as absent (0) or present (1). These molecular variations can be detected using molecular markers such as ISSR, which are powerful tools for detecting changes in DNA polymorphisms. Each locus's presence or absence was considered independent. Genetic diversity was determined by comparing banding patterns of three varieties (**Shaikhaldein *et al.*, 2022**). The polymorphism percentage was calculated by dividing the number of polymorphic loci by the total loci scored. Genetic similarities were evaluated using the Dice coefficient in STATISTICA 8. A clustering analysis was conducted to create a phylogenetic dendrogram with STATISTICA 8 software.

Micropropagation systems aim to produce genetically identical and stable offspring. Genetic fidelity studies are crucial for assessing the similarity between acclimated and the mother plant. In some plants, plants derived from organized meristems may not always exhibit genetic uniformity. Our data shows that the results indicated that more polymorphisms were similar among mother plants and acclimatized plants. Our conclusions also support the previous reports of many other researchers (**Kudikala *et al.*, 2020; Karwasra *et al.*, 2024**). The validation of genetic homogeneity in tissue-cultured plants using ISSR markers has been reported in many plant species, such as *Chlorophytum borivilianum* (**Karwasra *et al.*, 2024**), red-colored ginger (**George *et al.*, 2022**), *Solena amplexicaulis* (**Koppula *et al.*, 2023**), and *Adenium obesum* (**Dilna *et al.*, 2024**). The *in-vitro* cultured plants displayed 100% monomorphism between and within them, according to ISSR markers.

Table 1: ISSR primers with their nucleotide sequences, annealing temperature, temperature (°C), primer length, molecular weight (g/mol) and GC content (%).

Primers	Sequences (5′-3′)	Tm (°C)	Molecular weight g/mol	Primer length	GC content (%)
UBC825	ACACACACACACACT	54.9	4459.0	15	46.67
UBC826	ACACACACACACACACC	60.3	5046.3	17	52.94
UCB811	GAGAGAGAGAGAGAGAC	54.2	5366.6	17	52.94
UCB901	CACACACACACACACARY	57.2	4757.2	18	50.00
UBC835	AGAGAGAGAGAGAGAGYC	56.3	5366.6	18	52.94
UCB840	GAGAGAGAGAGAGAGATT	54.3	5685.8	18	44.44
UCB808	AGAGAGAGAGAGAGAGC	56.3	5366.6	17	52.94
UBC814	CTCTCTCTCTCTCTCAT	53.5	4998.3	17	47.06

Table 2: Effect of different concentrations of BA, 2iP, Zeatin and Kin on multiple shoot induction in cultured *Solenostemma argel*.

Multiplication					
Cytokinins (mg/L)		**No. of branches**	**No. of leaves**	**Length (cm)**	**Weight (g)**
Control	**0**	1.50 ± 0.22 ^{gh}	10.83 ±0.6 ^{h}	3.81 ±0.1249 ^{fg}	1.27±0.03 ^{g}
BA	**1**	3.16 ± 0.16 ^{cd}	15.66± 0.66 ^{f}	4.15 ±0.11 ^{defg}	1.55±0.02 ^{ef}
	2	2.16 ± 0.16 ^{efg}	12.167±0.6 ^{gh}	3.93 ±0.07 ^{efg}	1.41±0.02 ^{fg}
	4	2.50 ± 0.22 ^{def}	12.17±0.79 ^{gh}	4.43 ±0.27 ^{def}	1.48±0.07 ^{efg}
	8	2.00± 0.25 ^{fgh}	10.33 ±0.49 ^{h}	3.68 ±0.14 ^{g}	1.27±0.03 ^{g}
2iP	**1**	2.83 ± 0.16 ^{de}	18.67±0.88 ^{e}	5.26 ±0.46 ^{abc}	1.51±0.09 ^{ef}
	2	1.33 ± 0.21 ^{h}	11.83±0.87 ^{gh}	4.21 ±0.21 ^{defg}	1.5±0.08 ^{ef}
	4	2.00 ± 0.24 ^{fgh}	12.00±0.57 ^{gh}	4.18 ±0.20 ^{defg}	1.43±0.05 ^{fg}
	8	1.83 ± 0.16 ^{fgh}	11.33±0.49 ^{h}	3.71 ±0.22 ^{g}	1.58±0.07 ^{ef}
Kin	**1**	2.50± 0.22 ^{def}	24.33 ±1.02 ^{c}	5.48 ±0.35 ^{ab}	2.06±0.11 ^{abc}
	2	4.33± 0.42 ^{b}	29.00±0.73 ^{ab}	5.33 ±0.32 ^{ab}	2.15±0.09 ^{ab}
	4	4.33± 0.49 ^{b}	27.50±0.88 ^{b}	5.73 ±0.27 ^{a}	2.25±0.10 ^{a}
	8	3.00 ± 0.25 ^{d}	13.67±1.05 ^{fg}	4.55 ±0.24 ^{de}	1.85±0.09 ^{cd}
Zeatin	**1**	5.83 ± 0.25 ^{a}	29.66 ±0.49 ^{a}	5.33 ±0.32 ^{ab}	2.16±0.07 ^{a}
	2	4.16 ± 0.25 ^{b}	21.17±0.60 ^{d}	4.78±0.23 ^{bcd}	1.94±0.06 ^{bc}
	4	4.50 ± 0.42 ^{b}	20.33±0.88 ^{de}	4.81 ±0.13 ^{bcd}	2.04±0.07 ^{abc}
	8	3.83 ± 0.3 ^{bc}	18.5±0.67 ^{e}	4.6 ±0.20 ^{cde}	1.65±0.09 ^{de}
P-value		<0.001***	< 0.001***	< 0.001***	< 0.001***

Data are presented as mean ± SE. The *P* value was considered significant at <0.05. Different letters indicate significant statistical differences among groups within the same column.

Table 3: Impacts of IBA, IAA, and NAA concentrations on the number of roots, root length, and rooting percentage of *Solenostemma argel*

Rooting					
Auxins (mg/L)		**No. of root/Shoot tip (explants)**	**Length of longest root (cm)**	**Explant fresh weight**	**Rooting %**
Control	**0**	0.33±0.21 ^{f}	1.11±0.70 ^{e}	2.31±0.18 ^{ef}	6.66±4.21 ^{g}
IBA	**1**	2.00 ±0.25 ^{cd}	4.88±0.17 ^{a}	3.01±0.14 ^{cd}	30.00±2.58 ^{cde}
	2	2.66±0.21 ^{bc}	4.11±0.16 ^{ab}	3.25±0.15 ^{bc}	36.66±2.10 ^{bcd}
	4	5.16 ±0.30 ^{a}	3.38±0.22 ^{b}	4.08±0.09 ^{a}	65.0±5.00 ^{a}
	8	3.00 ±0.36 ^{b}	2.28±0.17 ^{cd}	3.75±0.26 ^{ab}	41.66±4.77 ^{b}
IAA	**1**	0.83±0.30 ^{ef}	2.20±0.70 ^{cd}	2.25±0.08 ^{ef}	15.0±5.00 ^{fg}
	2	1.66±0.21 ^{d}	2.48±0.08 ^{c}	2.25±0.12 ^{ef}	28.33±1.66 ^{de}
	4	2.5±0.22 ^{bc}	2.01±0.15 ^{cd}	2.98±0.24 ^{cd}	36.66±3.33 ^{bcd}
	8	1.66±0.33 ^{d}	1.96±0.12 ^{cde}	2.16±0.26 ^{f}	28.33±4.77 ^{de}
NAA	**1**	1.33±0.21 ^{de}	2.36±0.18 ^{c}	2.71±0.10 ^{de}	23.33±2.10 ^{ef}
	2	2.00 ±0.25 ^{cd}	2.11±0.12 ^{cd}	2.55±0.14 ^{def}	30.00±2.58 ^{cde}
	4	2.83±0.40 ^{b}	1.48±0.14 ^{de}	3.63±0.24 ^{ab}	40.00±5.16 ^{bc}
	8	1.50 ±0.22 ^{de}	1.65±0.12 ^{cde}	2.18±0.12 ^{f}	25.00±2.23 ^{ef}
P-value		< 0.001**	< 0.001**	< 0.001**	< 0.001**

Data are presented as mean ± SE. The *P* value was considered significant at <0.05. Different letters indicate significant statistical differences among groups within the same column.

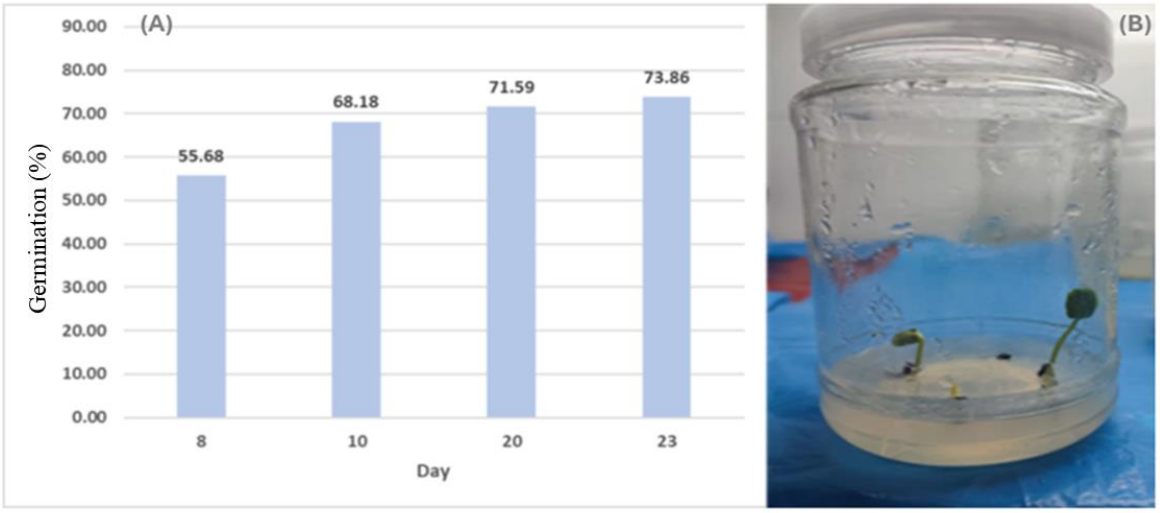

Figure 1: (A) The germination percentage of *in vitro* culture of *S. argel.* (B) The germinated seeds of *S. argel.*

Table 4: Impacts of MS supplemented with NAA + 2,4-D on the fresh weight, dry weight, colour, and shape of *S. argel*.

MS supplemented with	Fresh weight (g)	Dry weight (g)	Colour	Shape
NAA (2 mg/L)+ 2,4-D (1mg/L)	8.14±1.18[a]	0.56±0.11[a]	Greenish yellow	Compact nodular
NAA (1 mg/L)+ 2,4-D (1mg/L)	6.61±0.97[b]	0.41±0.09[b]	Greenish yellow	Compact nodular
NAA (1 mg/L)+ 2,4-D (0.5mg/L)	5.03±1.05[c]	0.32±0.07[c]	Greenish yellow	Compact nodular
P-value	<0.001***	<0.001***		

Tabel 5: The band scoring of different bands produced by eight ISSR primers in mother plant, and acclimatized *S. argel* plant and the associated polymorphism percentage.

Primers	ISSR 1	ISSR 2	ISSR 3	ISSR 4	ISSR 5	ISSR 6	ISSR 7	ISSR 8	Total	Average
Range of fragment size	357-697	337-814	290-830	292-877	337-445	390-1136	325-1022	342-833	290-1136	---
Mother plant	1	4	5	3	2	5	4	3	27	3.37
Adapted plants	2	3	4	3	3	4	4	3	26	3.25
No. of scorable bands	3	4	5	5	4	5	4	4	33	4.12
Monomorphic Fragments	0	0	1	2	2	3	2	1	16	2
Polymorphic Fragments	3	4	4	3	2	2	2	3	23	2.87
Unique loci	0	0	0	1	0	0	0	0	1	0.12
Polymorphism %	100	100	80	60	50	40	50	75	---	69.37

Table 6: The similar coefficient and genetic distance among mother plant, and acclimatized *S. argel* plant

Item	Mother	Adapted
Similarity coefficient		
Mother	1.00	0.92
Adapted	0.92	1.00
Genetic distance		
Mother	0.00	2.24
Adapted	2.24	0.00

Figure 2: (**A**) Effect of Zeatin (1 mg/L) on multiple shoot induction in cultured *Solenostemma argel*; (**B**) Effects of IBA (4 mg/L) enriched in MS medium on the number of roots, root length, and rooting percentage; (**C**) Effects of 2 mg/L NAA+1 mg/L 2,4D concentrations on callus formation; (**D**) Acclimatization of cultured *S. argel.*

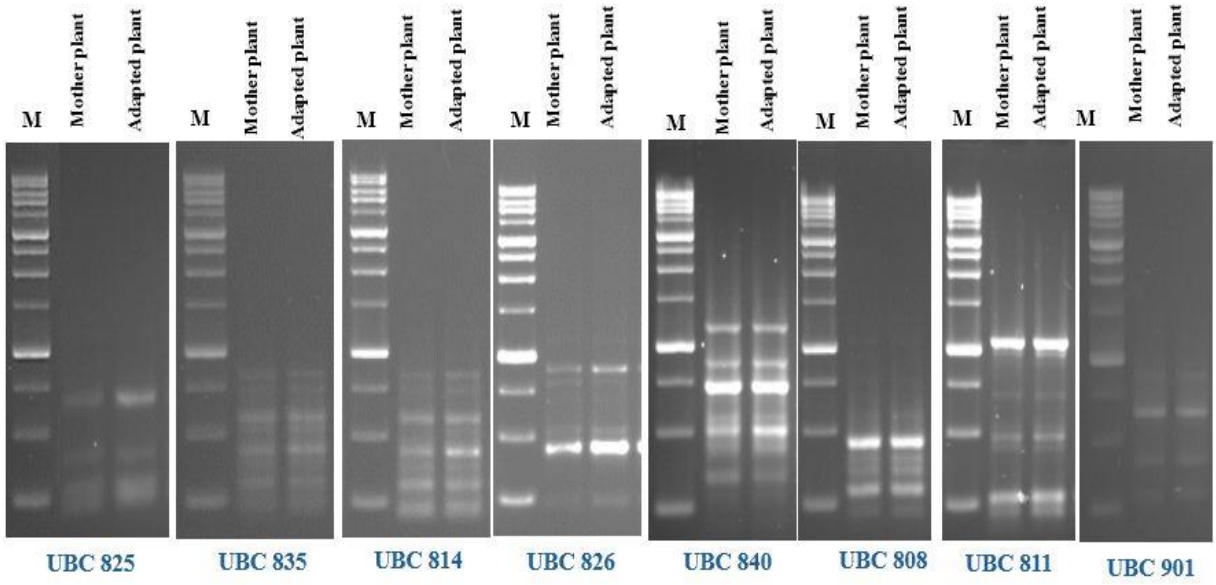

Figure 3: ISSR-PCR banding patterns of two *S. argel* cultivars. Lane M is showing a 100 bp ladder. This study used 8 types of ISSR to detect the variability of DNA fingerprint among five *S. argel* cultivars.

4. Conclusion:

This study provides *in vitro* propagation for critically endangered *S. argel*, especially those isolated from extreme environments such as Sinai. Collectively, the efficiency of micropropagation and rooting can be significantly improved by incorporating plant growth regulators such as zeatin or kinetin, along with auxins like IBA. This study could facilitate the sustainable exploitation of *S. argel* while contributing to its conservation strategies. Further study handling the quantification of the active metabolites is in progress.

Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

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