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In vitro propagation of *Alhagi maurorum* (Fabaceae)

Mustafa CUCE * [1]
ORCID: 0000-0001-7406-171X

[1] Department of Food Technology, Şebinkarahisar School of Applied Sciences, Giresun University, Türkiye

Abstract
Purpose: In this study, a contribution was made to a reliable and reproducible *in vitro* propagation protocol of the *Alhagi maurorum*, which belongs to the Fabaceae family and is of medicinal importance.
Method: Mature seeds were used as the initial material. In germination studies, MS medium without any plant growth regulator and MS containing 1.0 mg L^{-1} GA_3 were preferred as the basic medium. At the end of the germination culture period (4 weeks), MS medium supported with 1.0 mg L^{-1} GA_3 was found to be superior with 60% germination success. In shoot multiplication studies, nodal segments obtained at the end of the 4th subculture were used as explants, and MS basal medium was tested by combining 6-BA, kinetin, 2iP, zeatin and TDZ (1.0 mg L^{-1} separately with IBA (0.1mg L^{1}).
Findings: The highest mean values per explant for shoot number (4.75), node number (7.50), fresh weight (0.985 g), and dry weight (0.1171 g) were obtained from MS medium containing zeatin + IBA. MS medium supplemented with kinetin + IBA reached the highest mean shoot length with 69.26 mm. In terms of root induction, the highest rooting percentage (90.3%) was obtained in MS media enriched with 1.0 mg L^{-1} IBA after 4 weeks. This concentration of IBA yielded the highest root number and root length with 5.86 and 38.80 respectively, in MS medium. Rooted plants were initially acclimatized in a climate chamber and then carefully transferred to greenhouse conditions.
Conclusion: This propagation method can be considered for *ex situ* conservation and propagation of rare plant species of medicinal value.

Keywords: *Alhagi maurorum*, 2iP, zeatin, *in vitro* propagation

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Alhagi maurorum'un (Fabaceae) *in vitro* çoğaltılması

Özet
Amaç: Fabaceae familyasında yer alan ve tıbbi açıdan önemli bir tür olan *Alhagi maurorum'un* güvenilir ve tekrarlanabilir bir *in vitro* çoğaltma protokolüne bu çalışmada katkıda bulunulmuştur.
Metod: Başlangıç materyali olarak olgun tohumlar kullanılmıştır. Çimlendirme çalışmalarında temel besi ortamı olarak herhangi bir bitki büyüme düzenleyicisi içermeyen ve 1,0 mg L^{-1} GA_3 ile içeren MS tercih edilmiştir. Çimlendirme kültür süresi sonunda (4 hafta) 1,0 mg L^{-1} GA_3 ile desteklenen MS ortamı %60 çimlenme başarısı ile daha üstün bulunmuştur. Sürgün çoğaltma çalışmalarında 4. alt kültür sonunda elde edilen nodal segmentler eksplant olarak kullanılmış, 6-BA, kinetin, 2iP, zeatin ve TDZ (1.0 mg L^{-1}) IBA (0,1 mg L^{-1}) ile ayrı ayrı kombine edilerek MS temel besi ortamında test edilmiştir.
Bulgular: Eksplant başına ortalama en yüksek sürgün sayısı (4,75), nod sayısı (7,50), yaş ağırlık (0,985 g) ve kuru ağırlık (0,1171 g) değerleri zeatin + IBA içeren MS ortamından elde edilmiştir. Sadece kinetin + IBA ile desteklenmiş MS besi ortamı 69,26 mm ile en yüksek sürgün uzunluğuna ulaşmıştır. Kök indüksiyonu açısından, en yüksek köklenme yüzdesi (%90,3), 4 hafta sonra 1,0 mg L^{-1} IBA ile zenginleştirilmiş MS ortamında elde edilmiştir. Bu IBA konsantrasyonu, sırasıyla 5,86 ve 38,80 ile en yüksek kök sayısını ve kök uzunluğunu vermiştir. Köklenen bitkiler önce bir iklim odasında iklimlendirilmiş, ardından sera koşullarına aktarılmıştır.
Sonuç: Bu çoğaltma yöntemi, tıbbi değeri olan nadir bitki türlerinin *ex situ* muhafazası ve çoğaltılması için de düşünülebilir.
Anahtar kelimeler: *Alhagi maurorum*, 2iP, zeatin, *in vitro* çoğaltım

* *Corresponding author*: Tel.: +905373468681; Fax.: +94543101717; E-mail: mustafacuce@windowslive.com
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1. Introduction

Herbal natural products have served as essential medicinal and nutritional sources throughout human history due to their rich content of bioactive compounds [1, 2]. Researchers have increasingly tested natural products in search of alternative medicinal treatments, particularly for viral and microbial infections [2]. It is known that over 35,000 plant species are used for medicinal purposes in various cultures around the world. According to both recent and past researchers, terpenes, phenolic compounds, and alkaloids found in plants are natural antioxidants, most commonly found in medicinal plants in varying concentrations [3, 4]. One of the most important families of medicinal and aromatic plants is the Fabaceae family, which is represented worldwide by 550 genera and over 13,000 species with medicinal value. *Alhagi maurorum*, the only species of the *Alhagi* genus, is a rare halophytic medicinal plant that inhabits desert ecosystems [5].

Chemical studies have revealed that *A. maurorum* include many bioactive compounds such as alkaloids, coumarins, fatty acids, flavonoids, sterols, triterpenes, and vitamins, which are responsible for the plant's biological activities [6, 7]. These bioactive compounds grant *A. maurorum* biological activities such as antiulcerogenic [8], antiangiogenic [9], antioxidant, and anti-inflammatory effects [6]. Recent studies have revealed the presence of lupeol, a bioactive triterpenoid that plays an important role in lipid profile normalization, wound healing, protection against hypercholesterolemia, suppression of immune responses, and treatment of rheumatism [6] in *A. maurorum*. Furthermore, aqueous extracts from *A. maurorum* roots have been reported to aid in ureter expansion and thus help the removal of kidney stones [10]. Moreover, the essential oils found in this plant's leaves and stems are also well known for use in the cosmetics industry, aromatherapy, nutrition, perfumery, and as spice ingredients [11].

Today, there is a growing interest in the preference for herbal medicines and the commercialization of plant-based drugs. However, especially those valuable plant species are endangered due to anthropogenic pressures such as chemical pollution, habitat fragmentation, grazing, industrialization, road and dam construction, and activities related to nature tourism [2]. One of these species is *A. maurorum*. These unfavorable conditions prevent this species from sufficiently increasing the number of individuals in its natural populations, restricting its distribution and usage. Therefore, effective large-scale propagation of this rare plant species using various methods and the advantages of plant biotechnology is seen as a prerequisite for both conservation biology and meeting future pharmaceutical demands [12].

The development of *in vitro* propagation techniques offers significant opportunities for large-scale effective propagation and recovery of desired superior genotypes in a short time. These techniques have been widely applied to protect endangered plant species, obtain new genotypes, and propagate economically valuable plants. Furthermore, *in vitro* propagation of medicinal plants offers numerous advantages over traditional propagation methods, advantages that have significantly contributed to the growth of the pharmaceutical industry and continue to do so [13]. A review of the available literature reveals six different studies reporting *in vitro* propagation of *A. maurorum*. In these studies, only 6-benzyladenine (6-BA) and kinetin as cytokinin, and indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), and naphthalene acetic acid (NAA) as auxins were tested on the *in vitro* propagation of *A. maurorum* (12, 14, 15]. The aim of this study was to investigate the effects of five different cytokinins, two auxins and a control group on the *in vitro* propagation of *A. maurorum* and to develop a production method that will contribute to the conservation and propagation of rare medicinal plants.

2. Materials and methods

2.1. Plant material and seed germination

According to Pakistan's flora records, seeds of *A. maurorum*, the only species in the genus *Alhagi*, were collected in August and September 2010 near Malkani village in Badin district, Sindh Province, Pakistan. The identification of the species was aided by Prof. Dr. Tahir Rajpoot, former Director of the Institute of Botany, Sindh University, Jamshoro. The seeds were stored at + 4 °C until they were transferred to culture media for one year. To determine the optimal seed germination success of *A. maurorum*, full-strength Murashige and Skoog (MS) basal medium including vitamins [16], provided Duchefa Biochemie, Haarlem, The Netherlands, 2% (w/v) sucrose (Duchefa Biochemie), and 0.8% (w/v) phyto agar (Duchefa Biochemie) both without any plant growth regulator (PGR) and supplemented with 1.0 mg L^{-1} GA_3 were tested. Germination success was determined by calculating the germination percentage at the end of a four weeks incubation period.

2.2. Experimental

2.2.1. Sterilization

Before surface sterilization, mature seeds were thoroughly washed with tap water using a magnetic stirrer (Heidolph MR Hei-Tec, Schwabach, Germany) for 30 min at room temperatures. To improve germination success, seeds were subjected to hot water treatment at 70 °C for 5 min, followed by agitated at 500 rpm with 70% (v/v) ethanol (EtOH) for 30 seconds as a third pretreatment step. After these steps, ethanol was completely removed, and the seeds were carefully and thoroughly disinfected with 20% (v/v) commercial bleach (Domestos; active ingredient NaOCl, Unilever London, UK) for 10 min. Finally, to remove bleach residues, seeds were washed in a sterile biosafety cabinet (Biohazard, Italy) with sterile deionized water in three repetitions, each lasting at least 15 min. Approximately 15 mL of MS basal medium, including vitamins, was poured into 25×150 mm culture tubes (Sigma-Aldrich, St Louis, MO) for culturing. Subsequently, these sterile seeds were gently cultured in the abovementioned MS basal media without any plant growth regulator (PGR) and supplemented with 1.0 mg L^{-1} GA_3. The inoculated seeds were kept in a plant growth room during the germination period.

2.2.2. Shoot proliferation

In shoot multiplication experiments, nodal segments obtained from shoots grown *in vitro* over four subculture cycles after seed germination were used as explants. The MS basal medium, which contained vitamins such as glycine (2 mg L^{-1}), myo-inositol (100 mg L^{-1}), nicotinic acid (0.5 mg L^{-1}), pyridoxine HCl (0.5 mg L^{-1}), thiamine HCl (0.1 mg L^{-1}), 2% (w/v) sucrose (Duchefa Biochemie), and 0.8% (w/v) phyto agar (Duchefa Biochemie), was used as the basic medium for these studies. This basal medium was individually supplemented with 1.0 mg L^{-1} concentrations of commonly used cytokinins in micropropagation studies: 6-BA, kinetin, 2iP (6-(γ,γ-dimethylallylamino)-purine), thidiazuron (TDZ), or zeatin. To enhance cytokinin effectiveness, each of these five PGRs was also separately combined with 0.1 mg L^{-1} IBA and added to the MS basal medium. An MS medium without any PGR was used as a control. All PGRs used in the study were obtained from Sigma-Aldrich (St Louis, MO). Except for 6-BA and NAA, all other PGRs were added to the culture medium after autoclaving to avoid structural degradation. Sterile cellulose-based 0.22 µm Millex-GS filters (Merck Millipore, Ireland) were used to sterilize the PGRs added to the cooled (approx. 40 °C) medium. Media pH was adjusted to 5.8 using 1 N HCl or 1 N NaOH before autoclaving. All media and used experimental equipment were sterilized at 121 °C for 20 min at 105 kPa. Culture conditions were set at 24 ± 2 °C with a 16/8 h photoperiod under 50 µmol m^{-2} s^{-1} photosynthetic photon flux using cool daylight fluorescent lamps (Philips HO 49 W/840, Poland). The ideal subculture periods were set at 4 weeks after transferring explants to the culture media. Multiplication rates were calculated by evaluating the number of shoot, shoot length, number of nodes, fresh and dry weights of per explant. Furthermore, plant quality was evaluated using observational data such as internode length and shoot thickness.

2.2.3. Root induction

Healthy shoots of sufficient length (≥ 20 mm) obtained from the shoot multiplication media were subjected to rooting in MS basal medium supplemented with vitamins and different concentrations (0, 0.5, 1.0 and 2.0 mg L^{-1}) of IBA and NAA for four weeks. An MS medium without any auxin was used as the control. Root induction success of the shoots was evaluated in terms of rooting percentage, number of roots, and root length. Each experiment was conducted three times.

2.2.4. Acclimatization

To eliminate residual medium, roots of the plantlets removed from the rooting media were gently and carefully rinsed with running tap water without damaging the roots. The rooted microshoots were then transferred into a perlite medium inside 71 × 71 mm transparent, light-transmitting plastic containers supplemented with Hoagland supportive nutrient solution adjusted to pH 5.8. To control relative humidity, the containers were covered with another transparent plastic lid, and plants were subjected to an initial acclimatization process under the same growth chamber conditions for four weeks. During this period, the relative humidity was gradually decreased from 100% to 70% by Extech EN300 Digital Humidity and Temperature Meter. Subsequently, well-developed plants were transferred into trays containing a 4:1 peat: perlite mixture for further acclimatization in the same chamber conditions. Growth room conditions were set at 24 ± 2 °C with a 16/8 h photoperiod under 50 µmol m^{-2} s^{-1} light intensity using the same cold daylight fluorescent lamps used in the shoot multiplication study. The survival rate of the microshoots was recorded at the end of each four week acclimatization stage, and the surviving plants were transferred to a botanical garden.

2.2.5. Statistical analysis

The experiments (excluding acclimatization, which had four repetitions) were conducted using a completely randomized design with three replications. To minimize contamination during germination studies, one sterilized achene was placed in each culture tube, and 30 culture tubes were used for each replication. For shoot multiplication, five nodal explants were placed in one Magenta vessel, with six vessels per treatment. Each rooting experiment was conducted on 24 healthy shoots, with four shoots per Magenta vessel. For acclimatization studies, to minimize experimental error, 50 plantlets were evaluated for each treatment, and the experiment was repeated four times. Means of the data obtained from shoot proliferation and root induction studies were analyzed using one-way ANOVA via SPSS 21.0 (SPSS Inc., Chicago), followed by Duncan's Multiple Range Test (Duncan, 1955). This analysis method was chosen because the number of samples per treatment was equal (30 for shoot multiplication and 24 for rooting), data were homogeneous, and results followed a normal distribution suitable for parametric testing.

3. Results

3.1. Seed germination

Preliminary trials showed that MS medium without any PGR was insufficient for seed germination of *A. maurorum*. Therefore, MS medium without any PGR and supplemented with 1.0 mg L^{-1} GA_3 were individually tested for the germination of *A. maurorum* seeds due to the low germination percentage. Seed germination occurred in the absence of bacterial and fungal contamination. First germination was noted on the 6th day in the GA_3 supplemented medium, while it occurred on the 8th day in the control group. After four weeks, the MS medium supplemented with GA_3 achieved a 60% germination rate (Figure 1a) significantly outperforming the control medium (46%). The difference in germination success between the two media was statistically significant ($P < 0.05$).

3.2. Shoot proliferation

Among the six tested shoot proliferation media, the maximum number of shoots per explant (4.75 ± 0.86) was recorded in MS medium containing 1.0 mg L^{-1} 6-BA and 0.1 mg L^{-1} IBA. On the other hand, the control group and the TDZ + IBA treatment showed quite weak effects compared to the other treatments, with 1.56 ± 0.51 and 1.68 ± 0.48 shoots per explant, respectively. Statistical analysis revealed significant differences in shoot number among the tested cytokinins ($P < 0.05$), with the following order of effectiveness: zeatin > kinetin > 6-BA > 2iP > TDZ > control (Figure 1b-g). Independent of shoot number, the MS medium supplemented with 1.0 mg L^{-1} kinetin + 0.1 mg L^{-1} IBA showed the best performance in mean shoot elongation, reaching 69.26 ± 4.61 mm per explant (Table 1). Similar to the shoot number results, the control and TDZ + IBA treatments had the shortest shoot lengths, 25.44 ± 1.66 mm and 25.76 ± 2.34 mm, respectively. Significant differences were identified through statistical analysis ($P < 0.05$). When the five different cytokinins and one control medium were evaluated in terms of shoot length, the results were kinetin > 2iP > zeatin > 6-BA > TDZ > control (Table 1; Figure 1b-g). Regarding the number of nodes, no significant differences were observed. Both kinetin + IBA and zeatin + IBA combinations achieved 7.50 ± 0.97 and 7.50 ± 0.89 nodes per explant, respectively, while 2iP + IBA yielded 7.31 ± 0.87 for this parameter. No statistical difference was observed among these three treatments ($P < 0.05$). However, the other three media, 6-BA + IBA (2.38), control (2.25), and TDZ + IBA (2.19) formed a statistically distinct group with significantly lower values ($P < 0.05$).

Fresh and dry weight, which are critical parameters for evaluating the production of bioactive compounds in medicinal plants, were highest in the MS medium supplemented with 1.0 mg L^{-1} zeatin + 0.1 mg L^{-1} IBA compared to the other tested media. These two values were 0.985 ± 0.089 g (fresh) and 0.1171 ± 0.0291 g (dry) per explant in this medium. Although the 6-BA + IBA combination produced 0.831 ± 0.090 g (fresh) and 0.0732 ± 0.0075 g (dry) weight, these results were statistically inferior to zeatin + IBA ($P < 0.05$). TDZ + IBA and control treatments were also significantly less effective. The control group yielded the lowest values with 0.239 ± 0.017 g fresh and 0.0188 ± 0.0015 g dry weights. The order of effectiveness in fresh and dry weight values was consistent across treatments, and the ranking was zeatin > 6-BA > kinetin > 2iP > TDZ > control (Table 1; Figure 1b-g).

Table 1. Effects of four different cytokinins individually in combination with 0.1 mg L^{-1} IBA and a control group on shoot proliferation of *Alhagi maurorum*

Cytokinins (1.0 mg L^{-1})	Auxin (1.0 mg L^{-1})	Shoot number/ Explant	Shoot length (mm)	Node number/ Explant	Fresh weight (g)	Dry weight (g)
Control	-	$1.56^{e} \pm 0.51$	$25.44^{e} \pm 1.66$	$2.25^{b} \pm 0.58$	$0.239^{d} \pm 0.017$	$0.0188^{d} \pm 0.0015$
TDZ	IBA	$1.68^{e} \pm 0.48$	$25.76^{e} \pm 2.34$	$2.19^{b} \pm 0.66$	$0.268^{d} \pm 0.028$	$0.0195^{d} \pm 0.0029$
6-BA	IBA	$3.44^{c} \pm 0.73$	$28.53^{d} \pm 2.42$	$2.38^{b} \pm 0.50$	$0.831^{b} \pm 0.090$	$0.0732^{b} \pm 0.0075$
Kinetin	IBA	$3.94^{b} \pm 0.68$	$69.26^{a} \pm 4.61$	$7.50^{a} \pm 0.97$	$0.517^{c} \pm 0.070$	$0.0574^{c} \pm 0.0064$
2iP	IBA	$2.25^{d} \pm 0.68$	$66.65^{b} \pm 2.94$	$7.31^{a} \pm 0.87$	$0.474^{c} \pm 0.054$	$0.0539^{c} \pm 0.0058$
Zeatin	IBA	$4.75^{a} \pm 0.86$	$62.62^{c} \pm 2.91$	$7.50^{a} \pm 0.89$	$0.985^{a} \pm 0.089$	$0.1171^{a} \pm 0.0291$

Data were recorded 4 wk after the culture and represent a total of three replicates of 30 plants per treatment on MS for shoot proliferation. Values having the same letter(s) in the same column are not significantly different according to Duncan's multiple range test at $P < 0.05$ for shoot proliferation. TDZ = thidiazuron, 6-BA = 6-benzylaminopurine, 2iP = 6-(y,y-dimethylallylamino)-purine, IBA = Indole-3-butyric acid.

3.3. Root induction

The root induction culture conditions were similar to those used in shoot proliferation. No contamination or tissue browning was observed during the four week culture period. Although the desired level of root induction was not achieved in all treatments, the highest rooting rate (90.3%) was obtained on MS basal medium supplemented with 1.0 mg L^{-1} IBA. This result was significantly different from other treatments (Table 2; P < 0.05).

Table 2. The effects of two different auxin types on *in vitro* root induction of *Alhagi maurorum*

Auxin (mg L^{-1})					
Control	IBA	NAA	Rooting (%)	Root Number (No/Plant)	Root Length (mm)
0.0	0.0	0.0	0.0 ± 0.0f	0.0 ± 0.0e	0.0 ± 0.0e
	0.5		43.1 ± 2.4c	3.95 ± 0.42b	22.95 ± 2.55b
	1.0		90.3 ± 2.4a	5.86 ± 0.63a	38.80 ± 3.90a
	2.0		70.8 ± 4.2b	2.17 ± 0.31c	23.49 ± 2.24b
		0.5	50 ± 0.0e	1.83 ± 0.16d	19.14 ± 1.10c
		1.0	58.3 ± 4.2d	1.91 ± 0.15d	16.56 ± 1.68d
		2.0	65.6 ± 1.9c	1.76 ± 0.26d	17.81 ± 1.27d

Data were recorded 4 wk after the culture and represent a total of three replicates of 24 shoots per treatment on MS for root induction. Values having the same letter(s) in the same column are not significantly different according to Duncan's multiple range test at P < 0.05 for root induction. IBA = Indole-3-butyric acid, NAA = α- naphthalene acetic acid

Overall, the control treatment yielded no statistically significant rooting results, while IBA supplemented media proved more effective. Rooting percentages for MS media supplemented with 0.5 and 2.0 mg L^{-1} IBA were 43.1% and 70.8%, respectively. In addition, MS media containing NAA, rooting percentages were 50%, 58.3%, and 65.6% for 0.5, 1.0, and 2.0 mg L^{-1} NAA concentrations, respectively. Although NAA showed a significant rooting success depending on the increase in concentration, this success remained quite insufficient compared to IBA treatments. Regarding average root number and root length, the most effective result was obtained with 1.0 mg L^{-1} IBA with 5.86 ± 0.63 roots per shoot and 38.80 ± 3.90 mm root length (Figure 1h). For other concentrations of IBA, the same parameters were calculated as 3.95 ± 0.42 and 2.17 ± 0.31 roots per shoot, and 22.95 ± 2.55 mm and 23.49 ± 2.24 mm root length per shoot, at 0.5 and 2.0 mg L^{-1}, respectively. Among the three different NAA concentrations, the highest root number was obtained with 1.0 mg L^{-1} NAA (1.91 ± 0.15 roots per shoot), which was 67.4% less effective than the best IBA treatment. The longest roots under NAA treatments were observed at 0.5 mg L^{-1} NAA (19.14 ± 1.10 mm), again significantly inferior to the best IBA result.

Figure 1. Micropropagation of *A. maurorum*. (a) Seed germination in MS medium supplemented with 1.0 mg L^{-1} GA_3 (b-g) Shoot proliferation in MS medium individually supplemented with 1.0 mg L^{-1} of four PGRs; PGRs-free MS medium as control (b) control, (c) TDZ, (d) 6-BA, (e) kinetin, (f) 2iP, (g) zeatin, (h) Root induction on MS medium supplemented with 1.0 mg L^{-1} IBA after 30 days, Bars: (a) 2.5 cm, (b) 2.5 cm, (c) 2.5 cm, (d) 1.4 cm, (e) 2.8 cm, (f) 2.7 cm, (g) 2.5 cm, (h) 2.5 cm

3.4. Acclimatization

Approximately 200 healthy and green plantlets with well-developed roots were carefully transferred to pots containing 100% perlite supplemented with pH 5.8 Hoagland solutions. The pots were covered with plastic lids and kept in a controlled climate chamber under the same environmental conditions used during earlier stages. The lids were gradually opened over 30 days, reducing humidity from 100% to 70%, aiding adaptation to ex vitro conditions. The survival rate during this phase was calculated as 80%. Well-developed and sufficiently rooted plantlets that successfully completed the first acclimatization phase were then transferred to a 4:1 peat: perlite soil mix for another four weeks under the same conditions. The survival rate at this stage was 90%. Finally, the surviving plantlets were transferred to a botanical garden, where the survival rate remained at 90%.

4. Conclusions and discussion

Today, due to anthropogenic effects, major environmental changes such as wildfires, floods, earthquakes, and the construction of dams and roads have unfortunately accelerated the extinction processes of living organisms. In recent years, the rapid increase in human population has significantly elevated the use of bioactive compounds found in plants in both alternative and modern medicine. Unfortunately, this trend has also led to adverse effects such as urbanization, overharvesting, and uncontrolled collection of plant material [17, 18]. These negative effects on plant populations can be mitigated using *in situ* (on site) or *ex situ* (off site) conservation strategies. However, in situ methods alone are often inadequate due to unfavorable environmental factors. Plant tissue culture techniques are frequently considered as an alternative *ex situ* conservation method, especially for economically valuable or endangered plant species [2, 19]. In light of all this data, due to the limited number of *in vitro* propagation protocols in the literature and the challenges in sustaining natural populations, *A. maurorum* deserves priority in both *ex situ* conservation and *in vitro* propagation efforts.

Many researchers have used different types of explants such as seeds, leaves, nodal segments, young shoots, and shoot tips for *in vitro* propagation, and accordingly, they have used different sterilization agents and durations [2, 20]. Moreover, the timing of explant collection from natural populations directly affects both sterilization success and subsequent *in vitro* propagation studies [21]. This is mainly due to the sensitivity of the selected explant type to sterilizing agents and the potential risk of endophytic microorganisms contaminating the culture later. Although the use of seeds with low germination capacity as starting material in the current study may seem like a disadvantage, it actually presents several advantages such as long-term storage, easy accessibility, ease of transport, and convenience in choosing sterilization agents and duration. Therefore, it has also been preferred in previous studies [15, 22].

Different sterilization agents and durations have been tested in *in vitro* studies on other plant species [21, 23], but only $HgCl_2$ has been tested for *A. maurorum*. Additionally, no prior study has reported the use of GA_3, a well-known germination agent, for *A. maurorum* seed germination *in vitro*. The use of NaOCl for sterilization and the improved germination rates with GA_3 make this study significant. *In vivo* germination tests of *A. maurorum* have reported relatively low germination rates (24%) despite high viability with 95% [24]. In contrast, *in vitro* germination trials yielded higher success rates (63%), indicating that further efforts are needed to develop optimal conditions for seed germination. Our germination results (60%) align well with those findings.

Researchers often use MS as the basal medium in shoot multiplication studies of medicinal and economically valuable plants [19] and generally test cytokinins such as 6-BA, kinetin, 2iP, or zeatin at permanent or varying concentrations [2, 18, 19]. These cytokinins are often combined with a fixed auxin (IBA, NAA, or IAA) to enhance effectiveness [18]. In recent years, while the preference for single concentrations of PGRs has largely served conservation biology studies, as mentioned above, studies based on different concentrations of the same PGR have increasingly focused on bioactivity research. This study was designed to contribute to the conservation biology of the species. Furthermore, a single concentration approach was adopted because the aim was to determine the effect of PGRs such as TDZ and zeatin, which have not been previously tested on the species, against previously tested PGRs.

In *A. maurorum*, previous studies mainly used 6-BA or kinetin either alone or in combination with one of the above mentioned auxins in MS medium [17, 20]. Those studies suggested that 6-BA was more effective than kinetin. In contrast, the current study tested 2iP and zeatin in addition to these cytokinins, and found zeatin to be superior, distinguishing it from earlier research (Table 1). Agarwal et al. [17] reported that 2.0 mg L^{-1} 6-BA in MS medium yielded 4.7 shoots per explant. The shoot number obtained from the zeatin + IBA combination in our study was quite proportional to this, while their result obtained from the 6-BA + IAA combination (25 shoots per explant) was quite different. A study similar to the PGR types and concentrations used in this study was conducted on the *ex situ* preservation of *Tripleurospermum insularum* through *in vitro* production. Researchers obtained the most effective number of shoots per explant (3.33 shoots per explant) from MS medium containing 1.0 mg L^{-1} zeatin + 0.1 mg L^{-1} IAA [25]. These results highlight the varying effects of PGRs used in *in vitro* propagation studies depending on the plant species.

Similar trends were observed in shoot length. Agarwal et al. [12] reported that 2.0 mg L^{-1} 6-BA reached an average shoot length of 48.5 mm per explant in MS medium, and the same medium containing 0.5 mg L^{-1} 6-BA + 0.1

mg L^{-1} IAA reached an average shoot length of 70.7 mm. In this study, MS medium supported with 1.0 mg L^{-1} kinetin + 0.1 mg L^{-1} IBA was quite effective with 69.26 mm in terms of average shoot length per explant, and this result was consistent with the literature. Unlike this, researchers obtained the highest average shoot length per explant in *T. insularum* from MS medium supplemented with 1.0 mg L^{-1} zeatin + 0.1 mg L^{-1} IAA with 39.68 mm per explant [25]. These differences in shoot length reports support the idea that different PGR usage mentioned above can yield different results depending on the species. Although no comparative data on node number, fresh weight, or dry weight for *A. maurorum* were found in the literature, some reports indicate that 6-BA + NAA was effective for callus formation in MS medium [14]. The rooting success of *A. maurorum* differs from the results obtained by Inceer et al. [18] with *T. insularum* when compared with the literature. The researchers obtained a 100% rooting percentage in this species from MS medium containing 0.5 mg L^{-1} IAA. The highest number of roots was obtained from MS medium supplemented with 0.5 mg L^{-1} NAA, and the longest root length was obtained from PGR free MS medium. The fact that all these results were obtained from MS medium supplemented with 1.0 mg L^{-1} IBA in *A. maurorum* clearly demonstrates the differences.

In conclusion, this study aimed to develop an effective and rapid micropropagation and *ex situ* conservation protocol for *A. maurorum*, a rare and medically valuable halophytic species, through large-scale *in vitro* regeneration. Among the tested PGR concentrations, the combination of zeatin + IBA was found to be more effective for shoot number, node number, fresh weight, and dry weight. Kinetin + IBA outperformed other PGRs in shoot elongation. Rooting success with IBA reached approximately 90%. This protocol can serve as a valuable model not only for other endangered *Alhagi* species but also for other threatened and economically valuable plant species in terms of propagation, cryopreservation, and *ex situ* conservation strategies.

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