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In vitro mass propagation and conservation of a rare medicinal plant, *Zhumeria Majdae* Rech.f & Wendelbo (Lamiaceae)

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ABSTRACT

The monotypic *Zhumeria majdae* (Lamiaceae), an endangered medicinal and aromatic plant in the south part of Iran, has a low propagation rate in natural condition and therefore an efficient method for the *in vitro*-propagation is required. In the present study, the effects of source and position of the explants as well as various concentrations of 6-benzylaminopurine and kinetin on inhibition of hyperhydricity and *in vitro* mass propagation of the plant were investigated. The best shoot formation (1.9 ± 0.07) was obtained with a Murashige and Skoog medium fortified with 17.7 µM 6-benzylaminopurine. Regenerated shoots, elongated on the MS medium containing 2.2 µM 6-benzylaminopurine, were rooted on the different tested media, with the most abundant (68.6 ± 4.1%) and strongest roots obtained on half-strength medium without plant growth regulators. Hydrodistilled essential oils of *in vitro* regenerated plant were analyzed by gas chromatography–mass spectrometry and compared with the essential oil of wild plant. Thirty and twenty compounds representing 98.2% and 98.7% of the total oils were identified in the oils of wild plant and *in vitro* regenerated plant, respectively. The major essential oil components were linalool (31.7% and 41.7%), camphor (28.0% and 32.4%), limonene (4.6% and 8.3%), camphene (4.1% and 3.5%) and *E*-caryophyllene (1.0% and 2.7%) in the studied (wild plant and *in vitro* regenerated plant) essential oils. The *in vitro* regeneration system could be utilized for both conservation, large-scale multiplication and production of rich linalool essential oils of *Z. majdae*.

1. Introduction

In situ and *ex situ* strategies are well-known technologies for the plant diversity conservation, which have been widely used to establish effective conservation programs. One of the useful methods of the *ex situ* conservation for plant diversity is *in vitro* culture (Fay, 1994). Micropropagation of endangered plants can be beneficial for the rapid cultivation of this species, which exist in threatened habitats and have a limited reproductive capacity (Fay, 1992). *In vitro* propagation (IVP) protocols have already been established for different endangered medicinal plants such as *Siphonochilus aethiopicus* (Ngwenya et al., 2010), *Celastrus paniculatus* Willd. (Senapati et al., 2013) and *Thymus persicus* (Bakhtiar et al., 2014). IVP methods have also been established for the genus of Lamiaceae family such as *Lavandula* (Dias et al., 2002), *Salvia* (Bassolino et al., 2015) and *Thymus*(Marco-Medina and Casas, 2015).

Zhumeria majdae Rech.f & Wendelbo (Lamiaceae) commonly known as “Mohrekhosh” is an endangered perennial fragrant shrub, native to the southern tropical regions of Iran (Asareh, 2005; Jalili and Jamzad, 1999). This unique plant found in Hormozgan province in the south of Iran. The people use aerial part of this plant in folk medicine as anticonvulsant, anti-spasmodic and for dysmenorrhoea. Several reports are available about the antileishmanial and antiplasmodial (Moein et al., 2008), antibacterial (Mahboubi and Kazempour, 2009), anti-inflammatory (Sharififar et al., 2012), anticonvulsant (Mandegary et al., 2012) and antioxidant activities of the essential oil and extract of the plant. The antioxidant activity of the plant extract has been attributed to the presence and accumulation of phenolic compounds (Moein and Moein, 2010; Sharififar et al., 2008). The aerial parts of *Z. majdae* plants are harvested from their natural habitats, and also there is an increase in both internal and external market demands for their raw materials and valuable metabolites (Soltanipoor et al., 2007). Destructive harvesting procedures and over exploitation from its natural dry-arid habitats (Fig. 1) along with poor seed germination and low vegetative

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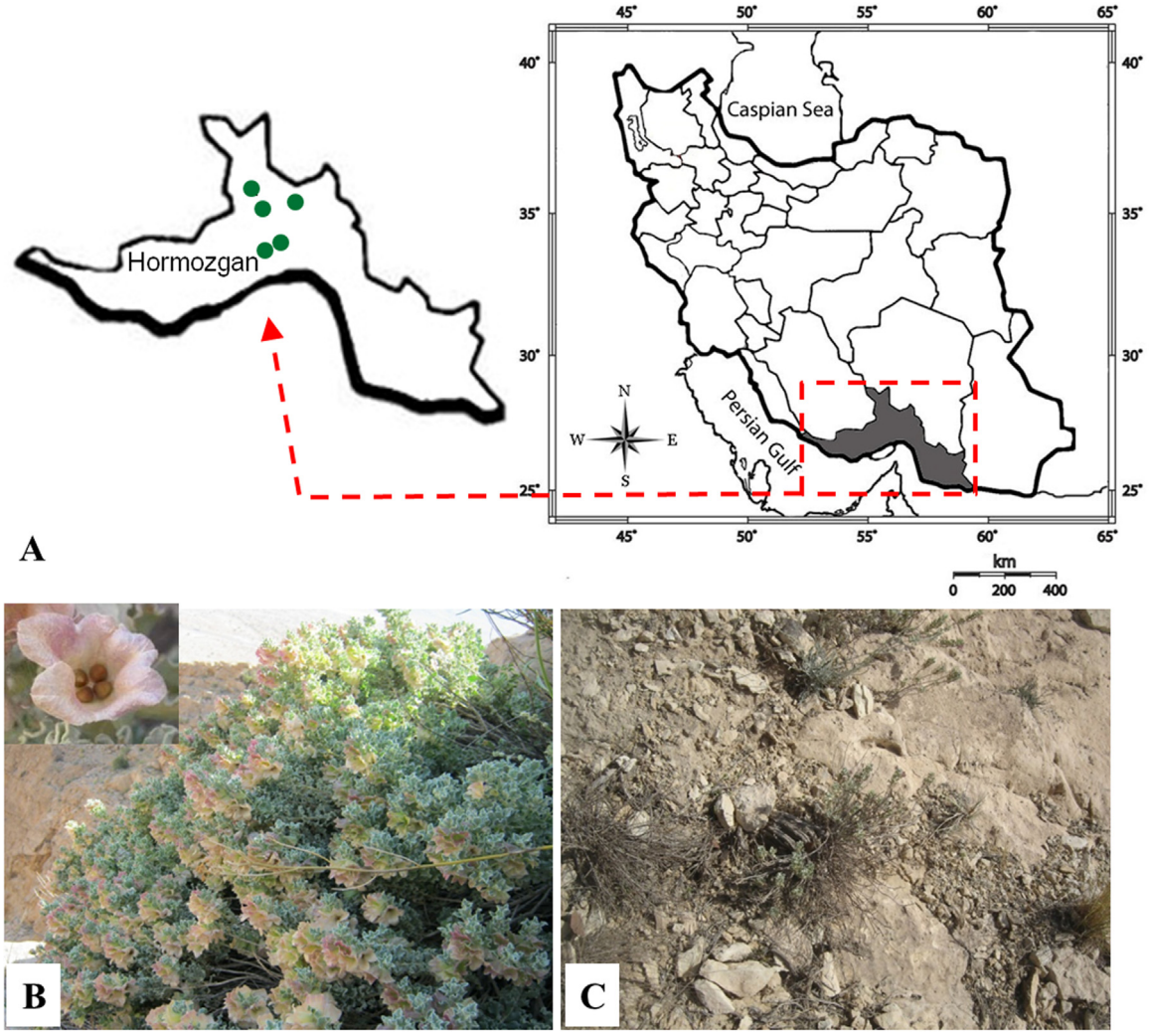

Fig. 1. *Zhumeria majdae*: (A) Distribution map, (B) Wild plant that served as seed source, (C) Habitat destruction due to climate change and over exploitation.

multiplication ratio lead to extinction of this species (Jalili and Jamzad, 1999) Therefore, a highly effective micropropagation technique would be useful for cultivation of the species. Although, seed germination and organogenesis of this plant from the callus cultures have already been reported (Aghazadeh and Behboodi, 2015), it is very necessary to introduce an effective protocol for the mass propagation of the plant due to protecting and preserving its natural habitats.

The development of micropropagation has been influenced by a wide range of difficulties such as discoloration of the medium, browning and hyperhydricity (vitrification) of the explant. Hyperhydricity is a limiting factor in the IVP of the plants which has previously been reported in the species of Lamiaceae family such as *Lavandula dentate* (Echeverrigaray et al., 2005), *Salvia officinalis* (Gostin, 2008) and *Salvia guaranitica* (Echeverrigaray et al., 2010). In hyperhydric plantlets, leaves have large vacuolated *meso*phyll cells, fewer stomata and less photosynthetic capacity, which decrease quantities of chlorophyll and protein (Kevers et al., 2004; Leshem, 1983; Sharma and Mohan, 2006). Their stem and leaves are often rigid, thick and breakable. During acclimatization, the ability of hyperhydric plants to grow normally is reduced and they have shown disorders (Yadav et al., 2003). Therefore, the present study was aimed to investigate the effects of various concentrations of plant growth regulators (PGRs), explant source (*in vitro* grown seedlings, pot-grown plants and wild plants) and explant type (shoot tips and nodal segments associated to their position along the shoot axis) on the IVP of *Z. majdae*, especially to control of hyperhydricity. The essential oil of *in vitro* regenerated plant (IVRP) was also analyzed and compared to that of wild plants (WP) in order to ensure that biosynthetic ability was not altered as a consequence of the regeneration protocol. Our findings could be utilized for *in vitro* and *ex vitro* conservation, large-scale multiplication and production of rich linalool essential oils of the plant.

2. Materials and methods

2.1. Plant materials and chemicals

Mature seeds of *Z. majdae*, collected from the plants growing in the natural habitat were obtained from the Department of Natural Resources, Yazd University. In addition, mature plant shoot segments (10–12 cm) were collected from wild growing plants in the Geno Mountain (27° 23′ 10″ N, 56° 11′ 55″ E at an altitude of 800 m), Bandar-Abbas, Hormozgan Province in the south of Iran (Fig. 1). One-year-old pot-grown plants were also used to evaluate the explant source on the initial *in vitro* establishment. A voucher specimen of the plant has been deposited at the Herbarium of Medicinal Plants and Drugs Research Institute (MPH), Shahid Beheshti University, Tehran, Iran.

Basal media compositions such as salts, vitamins, sucrose, agar, and plant growth regulators (PGRs) were bought from Merck (Darmstadt, Germany) and Sigma (Sigma-Aldrich Corporation, MO, USA) company.

2.2. Surface-sterilization procedures, seed germination and initial culture establishment

Shoot segments (4–5 cm length) were washed under running tap water for 30 min to remove adhering dust and any other foreign material, pre-soaked in running tap water for 5 min with a few drops of mild detergent. After washing (3 times) with distilled water, they were surface sterilized by immersing in 70% ethanol for 30 s, followed by 10 min in 1% sodium hypochlorite containing one drop of Tween 80 (Merck, Darmstadt, Germany) with continuous shaking under laminar air flow cabinet, then washed five times with sterile distilled water to remove traces of NaOCL. The surface sterilized explants were resized to lengths of approx. 3–4 cm, were cultured on basal MS (Murashige and Skoog, 1962) medium. The seeds were washed under running tap water for 3 h. After removing their mucilage coating by hand as well as removing hollow and insect-damaged seeds, soaked in 70% (v/v) ethanol for 30 s followed by disinfecting in 2% NaOCL for six min under aseptic conditions, then rinsed 4 times in sterilized distilled water. The seeds were then immersed in sterile distilled water (20 ml in 100 ml Erlenmeyer flasks) on a rotary shaker (100 rpm) in the dark. The seeds were allowed to germinate (4 weeks) and then inoculated onto MS medium (under cool fluorescent lights) for further growth and development. The number of germinated seeds was counted at the end of the period, and they were transferred onto fresh initiation medium every four weeks for three passages. 90 days after sowing, shoot tips and single nodal segments isolated from one seedling were cultured in MS medium fortified with 4.4 μM 6-benzylaminopurine (BAP) to obtain enough shoots for the subsequent proliferation evaluations.

2.3. Culture conditions

Cultures were grown in 200 ml glass jars closed with transparent polycarbonate caps, with 40 ml culture medium. All of the media except ½ MS (15 g l^{-1} sucrose), were supplemented with 30 g l^{-1} sucrose (Merck, Darmstadt, Germany) and solidified with 9 g l^{-1} agar (Merck, Darmstadt, Germany). The pH of the culture medium was adjusted to 5.8 with 1 N HCl or 1 N NaOH, before addition of agar and autoclaving at 121 °C for 20 min. Cultures were incubated under a 16-h photoperiod provided for 30 days by cool-white fluorescent lamps (Philips, 58 W, Holland) at a photon flux density of 40 μ mol m^{-2} s^{-1} at 23 ± 2 °C. Each glass jar was considered as an experimental unit and the experiment done at least three times with 10 replicates per treatment. The cultures were subcultured at regular intervals of 30 days on fresh MS medium.

2.4. Shoot multiplication

Initially, in order to assess the effect of PGRs on the proliferation of *Z. majdae* shoots, MS medium supplemented with different concentrations of BAP and KN was used, and the effective concentrations was then selected. In addition, the effect of source and position of the explants on the control of hyperhydricity and *in vitro* shoot multiplication of the plant were studied. *In vitro*- grown seedlings (IVGS), wild-grown plants (VGP) and pot-grown plants (PGP) of *Z. majdae* were the source of explants. Shoot tip (St) and single nodal segments based on their position along with the length of shoot source (n2, n3, n4, and n5) considered as the position of the explants (Fig. 2*A*). Selected explants were cultured in a row (Fig. 2*B*) on the MS medium supplemented with various concentrations of BAP (2.2, 4.4, 8.9, 17.7, 31.1 and 71.0 μM) and KN (2.3, 4.6, 9.3 and 18.6 μM). Shoot height (cm), number of shoots per explant, number of nodes per shoots, and percentages of hyperhydric and morphological abnormal shoots were recorded after 4 weeks of culture.

2.5. Shoot elongation, induction of rooting and acclimatization

Following the shoot proliferation stage, well-grown shoots were isolated and transferred to MS medium supplemented with 2.2 μM BAP in order to promote shoot elongation. This concentration of BAP has been optimized during preliminary experiments. Elongated shoots harvested at the end of the proliferation stage were transferred to half or full-strength MS medium supplemented or not with indole-3-acetic acid (IAA) (1.1, 5.7 or 14.3 μM) and indole-3-butyric acid (IBA) (0.98, 4.9 or 12.3 μM) for rooting. Shoots with at least one adventitious root (0.3 cm in length) were recorded. The rooting percentage, mean root number and mean root length (cm) per plantlet were measured. Plantlets with well-developed roots (more than two roots of at least 3.5 cm in length), grown on PGR-free ½ MS medium, were selected for acclimatization and transplanted to plastic pots containing a mixture of *Pistacia atlantica* and *Juniperus* forest duff. The pots were shielded with transparent polyethylene covers to initially maintain the plantlets at high humidity and were then placed in a plant growth chamber. The chamber was set at 20 ± 2 °C with 70% relative humidity (RH) under a 14 h photoperiod. The pots were irrigated with tap water every 7 days. After 4 weeks, they transferred to a greenhouse for further growth. The Plantlets with good growth were further kept in the greenhouse under normal day length at 22 ± 2 °C during the day and 18 ± 2 °C at night. Finally, the plantlets were transferred to the field for further growth.

2.6. Essential oil isolation and analysis

The essential oil of shade-dried aerial parts (30 g) of fully rooted *in vitro* regenerated plant (IVRP) and leaves of the wild plant (WP) were isolated by hydro-distillation for 3 h using a Clevenger-type apparatus according to British Pharmacopoeia (1993). The essential oils were dried over anhydrous sodium sulfate (Na_2SO_4) and stored in a sealed glass vial at −18 °C until analysis. Gas chromatography-flame ionization (GC-FID) and GC–mass spectrometry (GC-MS) analyses as well as identification and quantification of the oils components were carried out as described previously (Raeisi et al., 2015).

2.7. Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) to assess treatment differences using the SAS statistical package for Windows (Version 9.1.3). Analysis of mean (ANOM) was determined using Duncan's New Multiple Range Test ($P \leq 0.05$).

3. Results and discussion

3.1. Seed germination and establishment of explants

As the extreme aim of this work is the conservation of *Z. majdae*, seeds are the ideal beginning material to initiate *in vitro* cultures because they allow the maintenance of a wider genetic base (Fay, 1992). *Z. majdae* seeds produce a thick layer of mucilage around the pericarp within minutes after hydration. The mucilage was removed by hand. Mucilage removal did not inhibit seed germination under ideal laboratory conditions. After mucilage removing, 70% of the collected seeds from the habitat were empty. The aseptic seeds were incubated in the distilled sterile water on a shaker until to let primary roots reach 1–2 cm in length. 31.9% seeds germinated within 4 weeks of inoculation in sterile water. Of course, most of the seeds germinated within the first two weeks (data not shown). The germinated seeds transferred gradually to MS medium. The seeds developed into plantlets (5–6 cm) consisting of 6–7 pair of leaves in MS medium within 60 d of germination. *Z. majdae* seedling exhibited a strong apical dominance that resulted in slow growth of axillary shoots. The seedlings (11.0%) also showed symptoms of hyperhydricity. After four weeks, the contamination (%) of explants from 1-year old pot-grown and wild mature *Z. majdae* plants cultured on PGR-free MS medium were 12.0% and 91.0%, respectively. Stock plants grown under greenhouse conditions give rise to better results than the ones grown in wild conditions. Also,

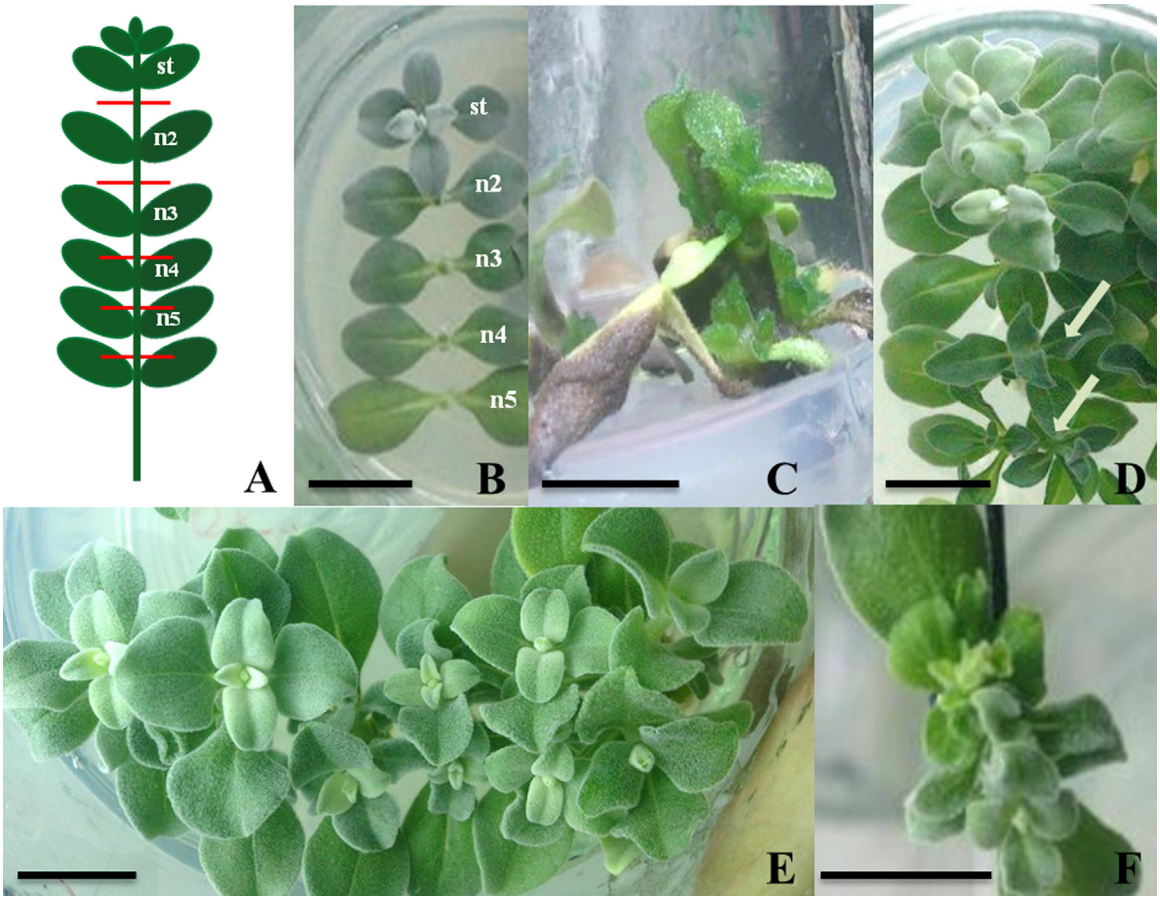

Fig. 2. *In vitro* clonal propagation of *Zhumeria majdae*: (A) Shoot tip (st) and single nodal segments based on their position along with the length of shoot source (n2, n3, n4, and n5) that considered as the position of the explants, (B) St and single nodal explants cultured on the MS medium, (C) Hyperhydricity and leaf abscission in nodal explant from wild mature plants, (D) Hyperhydricity in shoots that originated from basal segments, (E) Regenerated plantlets from nodal explants on the medium supplemented with BAP (17.7 µM), (F) Morphological abnormality of regenerated shoots from nodal explant on medium supplemented with BAP (71.0 µM). Bar = 1 cm. (G, H) Transplanted plant in pots after 1 month. (I)Regenerated plantlets of *Z. majdae* after 6 months of acclimatization. (A – F: bar = 1 cm and G-I: bar = 3 cm).

89.0% and 100% of decontaminated explants from pot-grown and wild plants became hyperhydric, respectively (Fig. 2C). The term hyperhydration, previously known as vitrification, refers to the morphological, physiological and metabolic derangements frequently affect in herbaceous and woody plants during their *in vitro* culture (Debergh et al., 1992). Shoots did not develop, only some shoot growth was observed occasionally. In order to reduce the occurrence of hyperhydricity, adding 2.0 and 4.0 mg/l $AgNO_3$ to the MS medium, decreasing of the NH_4NO_3 level (5.1 and 10.3 µM) and vessel aeration using caps with cellulose acetate filter (0.45 µm pore size, 7 mm diameter) were examined. These treatments showed variable results on the control of hyperhydricity (data not shown). Finally, these explants failed to use to further studies. Our results showed that the source of explants has a significant influence on the regeneration ability, which could be a kind of age-related response. The explants taken from *in vitro* grown seedlings were found to be best starting material for *Z. majdae* micropropagation. Plant material is key factor for the efficacy and success of tissue culture studies (Tisserat, 1985). Plant micropropagation depends upon the interaction of several endogenous and exogenous factors. The physiological status of an explant, concentration of plant growth regulators in culture medium and their interactions are of the major agents among these factors (Litz et al., 2005; Palanisamy and Kumar, 1997). The position of the explants on mother plant and their ontogeny significantly affect the *in vitro* micropropagation. Due to the difference in age, endogenous metabolic profile and differential genome, different explant tissues have various growth potential.

3.2. Shoot multiplication

All data on different growth parameters were noted after 4 weeks of culture initiation and then the regenerated shoots transferred to the new medium for further elongation. If the shoots were kept longer time (more than 4 weeks) in culture medium and not subcultured, elongation continued but severe hyperhydricity symptoms appeared or the nodal segments taken from these shoots were more vulnerable to hyperhydricity. Also Benson et al. (2000) reported that the probability of leaf hyperhydricity and abnormal growth increased in *Primula* plantlets when the plantlets were not subcultured to new a medium. According to Table 1, the main effect of bud positions regardless of cytokinin concentrations showed that the origin of the explant influenced the development of hyperhydricity and proliferation. From normal shoots, basal nodal segments formed a higher percentage of hyperhydric shoots than upper nodes (Fig. 2D). These results are in agreement with those obtained by Zimmerman et al. (1991) for *Petunia,* who found that *in vitro* growth of explants can be affected depending on the place from where they are excised. They reported that the origin of nodal explants influenced hyperhydricity and basal segments formed a higher percentage of hyperhydric shoots than the upper nodes. St and n2 often gave healthy shoots while the number of shoots per explant was (1.5 and 1.6 respectively) lower than n3, n4 and n5 (1.8, 1.9 and 2.0 respectively) segments (Table 1). The greater responsiveness of lower nodal explants over st and n2 can be attributed to the absence of apical dominance and the presence of axillary buds at a more advanced stage

Table 1
The effect of explant position on the number of shoots, shoot length, number of nodes and percentage of hyperhydric *Z. majdae in vitro* propagated shoots 4 weeks after culture initiation on MS medium containing various concentrations of BAP and KN.

Explant position	Frequency (%)	Number of shoots/ explant (no. ± SE)	Shoot length (cm ± SE)	Number of nodes /shoot (no. ± SE)	Hyperhydric shoots (%)
st	100 a	1.5 ± 0.16 b	1.0 ± 0.06 a	2.0 ± 0.10 a	0.86 ± 0.13c
n2	97.7 a	1.6 ± 0.08 b	0.84 ± 0.05c	1.3 ± 0.08c	3.1 ± 0.32c
n3	99.4 a	1.8 ± 0.12 a	0.96 ± 0.05 ab	1.4 ± 0.07 bc	5.1 ± 0.45 bc
n4	98.0 a	1.9 ± 0.11 a	1.0 ± 0.05a	1.6 ± 0.09 b	8.1 ± 0.48 b
n5	99.2 a	2.0 ± 0.12 a	0.89 ± 0.05 bc	1.4 ± 0.09 bc	22.4 ± 0.87 a

Different *lower case letters* indicate significant differences between explant position at $P \leq 0.05$, SE standard error.

Table 2
The effect of the various concentrations of BAP and KN on the number of shoots, shoot length, number of nodes and percentage of hyperhydric *Z. majdae in vitro* propagated shoots 4 weeks after culture initiation on MS medium.

Cytokinin	Frequency (%)	Number of shoots/ explant (no. ± SE)	Shoot length (cm ± SE)	Number of nodes /shoot (no. ± SE)	Hyperhydric shoots (%)
BAP(μM)					
2.2	96.5 a	1.4 ± 0.06 def	1.4 ± 0.05a	2.1 ± 0.11 a	8.6 ± 0.95a
4.4	98.8 a	1.5 ± 0.08 def	1.3 ± 0.04 a	2.0 ± 0.08 a	11.0 ± 1.1 a
8.9	100 a	1.6 ± 0.08 cd	1.1 ± 0.06 b	1.9 ± 0.11 ab	7.6 ± 1.1a
17.7	100 a	1.9 ± 0.07c	1.1 ± 0.04 b	1.8 ± 0.06 b	9.6 ± 0.95a
31.1	100 a	2.2 ± 0.09 b	0.76 ± 0.03 d	1.5 ± 0.07c	11.4 ± 1.1 a
71.0	100 a	3.4 ± 0.20 a	0.56 ± 0.04 e	0.85 ± 0.08 e	8.4 ± 0.69a
KN(μM)					
2.3	96.2 a	1.3 ± 0.05 f	0.86 ± 0.05 cd	1.4 ± 0.11c	8.8 ± 0.80a
4.6	100 a	1.4 ± 0.09 def	0.92 ± 0.05c	1.4 ± 0.09c	5.6 ± 0.76 a
9.3	98.8 a	1.4 ± 0.06ef	0.73 ± 0.05 d	1.1 ± 0.11 d	4.2 ± 0.59 a
18.6	98.7 a	1.6 ± 0.09 de	0.78 ± 0.04 d	1.3 ± 0.11 cd	3.7 ± 0.50 a

Different *lower case letters* indicate significant differences between cytokinins concentration at $P \leq 0.05$, SE standard error.

of development. The highest adventitious shoots (1.0 cm) were proliferated from st and n4 explants. Maximum number of nodes per shoot (2.0) was observed in shoots obtained from the st explants. On the other hand, various concentrations of BAP and KN were investigated on the shoot proliferation. Without considering the explant type, BAP was better than KN on the shoot proliferation. With increasing the concentration of cytokinins, the number of shoots per explant was increased but the height of plantlets was reduced (Table 2). The maximum number of shoots (3.4 shoots/explant) was obtained on the medium containing 71.0 μM BAP, while the best shoot proliferation rate (1.9 shoots/explant) was obtained in 17.7 μM BAP (Fig. 2*E*). The percentage of hyperhydricity was raised during the elongation phase in the shoots originating from the medium containing 31.1 and 71.0 μM BAP. Furthermore, morphological abnormality of regenerated shoots was observed at high concentrations of BAP (Fig. 2*F*). Maximum number of node per shoot and shoot length was recorded in MS medium containing 2.2 (2.1, 1.1 cm) and 4.4 (2.0, 1.3 cm) μM BAP, respectively. BAP in lower concentration had less effect on apical dominance and, therefore, length of shoots increased. The effect of different concentrations of BAP and KN with explant positions on shoot proliferation of *Z. majdae* as shown in Fig. 3.

3.3. Rooting of shoots

Rooting of elongated shoots was performed on half and full strength MS medium hormone-free or MS medium supplemented with IAA and IBA at various concentrations. The highest rooting rate (68.6%) was observed on ½ MS medium hormone-free whereas MS medium hormone-free yielded 36.3% (Table 3). In the other species belonging to the Lamiaceae family such as *Teucrium stocksianum* (Bouhouche and Ksiksi, 2007), *Lavandula vera* (Andrade et al., 1999) and *Lavandula viridis* (Dias et al., 2002), it was observed that decreasing the macronutrient concentration improved root formation. These results differ from those observed by Lê (1989) and Ozudogru et al. (2011), where they obtained 100% rooting of regenerated *T. vulgaris* on MS medium. In MS medium supplemented with auxin, the only low concentration of IBA (0.98 μM, 42.3%) was better than hormone-free MS medium. However, it was lower than ½ MS hormone-free. Baker and Wetzstein (1994) reported that a higher concentration of auxin block the regeneration process because it stimulates a higher level of degradative metabolites in tissue. For both IAA and IBA at higher concentrations, root development was accompanied with callus formation at the base of shoots, and they were thick, fragile and easily broken during transfer of plantlets to the soil, making their acclimation more difficult (Fig. 4). In term of root length, there was no significant difference between tested treatments, although a maximum number of the root (2.6 ± 0.32) and rootlet (16.6 ± 1.4) occurred when the explants were rooted in the presence of highest concentration of IBA (12.3 μM).

3.4. Acclimatization

Well-rooted plantlets were gently washed with tap water to remove adhered agar and traces of medium to avoid contamination, and then plantlets were transferred to the small pots. In the first week of transplantation, the plantlets were kept covered continually in a polyethylene cover to provide high humidity and allow sufficient light. After one week, the polyethylene cover was removed periodically and progressively whenever cover appeared to be wet (Fig. 5*A*). The polyethylene covers were entirely removed after 4 weeks of hardening. The survival rate was 80.0% and the appearance of acclimatized plants was normal without any morphological abnormalities (Fig. 5*B*). One month after the beginning of acclimation, rooted plantlets were transferred to the field for further growth. After 6 months, fully-acclimatized *Z. majdae* plants were phenotypically indistinguishable from wild plants (Fig. 5*C*).

3.5. Essential oils analysis

The hydrodistillation of the aerial parts of WP and IVRP gave colorless essential oil in 8.6% and 5.5% (w/w based on dry weight) yield, respectively. The essential oil content of the plant has been previously reported in ranging from 5.3% to 11.1% at different growth stages in

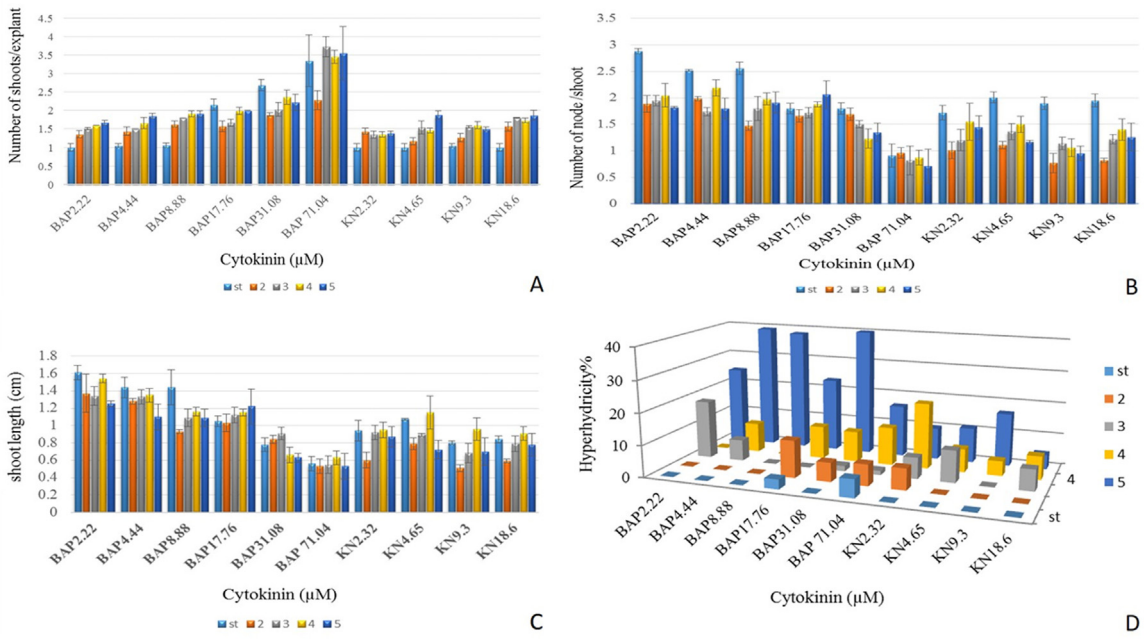

Fig. 3. The effect of BAP and KN concentrations and the position of explants on the number of shoots (A), number of nodes (B), length of shoots (C) and hyperhydric shoots (D) produced from *Zhumeria majdae*.

which maximum content was recorded after flowering stage (Soltanipoor et al., 2007; Arman et al., 2009). The chemical composition of the essential oils was analyzed by GC and GC-MS. Retention indices (RI) and percentage of the compounds identified in the oils are listed in the Table 4 in order of their elution on the DB-5 column. Totally, thirty and twenty compounds were identified and quantified in WP and IVRP, representing 98.2% and 98.7%of the total oil, respectively. The major components were linalool (31.7% and 41.7%), camphor (28.0% and 32.4%), limonene (4.6% and 8.3%), camphene (4.1% and 3.5%) and *E*-caryophyllene (1.0% and 2.7%) in the studied (WP and IVRP) essential oils. The classification of the identified compounds, based on the functional group was summarized at the end of Table 4. As can be observed, the oxygenated monoterpenes (77.4% and 78.5%) followed by monoterpene hydrocarbons (13.8% and 16.1%) were the main group of compounds in the WP and IVRP essential oils, respectively. Based on the obtained results, linalool and camphor were the main components in the *Z. majdae* essential oil. Linalool is one of the most useful monoterpene alcohols for the perfumery industry as well as for synthesis route to vitamin E (Frighetto et al., 1998). Furthermore, this compound has exhibited antinociceptive (Batista et al., 2008), anticonvulsant (Elisabetsky et al., 1999) and sedative activities (Sugawara et al., 1998). Camphor has a counterirritant, rubefacient and mild analgesic action, and is a major component of liniments for relief of fibrositis, neuralgia and similar conditions. It can be used as a mild expectorant; if ingested, camphor has irritant and carminative properties (Reynolds, 1989). The main plant part used in traditional medicine is the aerial part of the wild plant. We have taken the IVRPs to see the differences in the composition of essential oil with that of WP. Similar works on the essential oil composition of *in vitro* and *in vivo* medicinal plants has been reported (Chebel et al., 1998; Fortunato and Avato, 2008). For example, Fortunato and Avato (2008) have been reported that the IVRP of *Origanum vulgare* L. ssp. *hirtum* is capable of producing essential oil rich carvacrol as well as WP.

4. Conclusions

The present work establishes an efficient method to control hyperhydricity and *in vitro* mass propagation of *Z. majdae*, a monotypic endangered medicinal plant, from different explants by application of exogenous BAP and KN. The aseptic seeds of the plant germinated in the distilled sterile water on a shaker within four weeks. Upper nodal explants taken from *in vitro* grown seedlings were found to be the best starting material for *in vitro* regeneration. From normal shoots, basal nodal segments formed a higher percentage of hyperhydric shoots

Table 3
The effects of MS medium with or without IAA and IBA on rooting of *in vitro* regenerated *Z. majdae* shoots after 4 weeks of culture.

Basal medium	Auxin (μM)	Rooting (%)	Mean number of roots /shoots (cm ± SE)	Length of longest root (cm ± SE)	Mean number of rootlets/ shoots (cm ± SE)	Mean rootlet length (cm ± SE)	Callus formation	Fragile roots
½ MS	0	68.6 a	2.3 ± 0.13 ab	3.9 ± 0.21 a	10.7 ± 1.2 cd	1.3 ± 0.06c	-	-
MS	0	36.3 bc	2.2 ± 0.13 ab	4.6 ± 0.28 a	7.1 ± 0.57 d	2.0 ± 0.15 ab	-	-
	IBA							
	0.98	42.3 b	1.9 ± 0.21 ab	4.2 ± 0.31 a	6.8 ± 0.73 d	1.5 ± 0.17 bc	-	-
	4.9	36.6 bc	1.6 ± 0.19 b	4.6 ± 0.19 a	15.4 ± 1.9 ab	1.5 ± 0.14 bc	+	+
	12.3	33.3 bc	2.6 ± 0.32 a	4.1 ± 0.24 a	16.6 ± 1.4 a	2.0 ± 0.12 ab	++	++
	IAA							
	1.1	27.0 bc	2.0 ± 0.28 ab	4.6 ± 0.55 a	8.2 ± 1.1 cd	1.4 ± 0.17c	-	-
	5.7	27.6 bc	1.5 ± 0.14 b	4.5 ± 0.32 a	6.6 ± 1.1 d	1.3 ± 0.18c	+	+
	14.2	21.0c	1.5 ± 0.39 b	4.0 ± 0.56 a	12.1 ± 1.9 bc	2.3 ± 0.30 a	++	++

Different *lower case letters* indicate significant differences between medium and auxins concentration at $P \leq 0.05$, SE standard error, Callus formation (+: Callus induction, -:No callus formation), Fragile roots (+: Fragile, -: Not fragile).

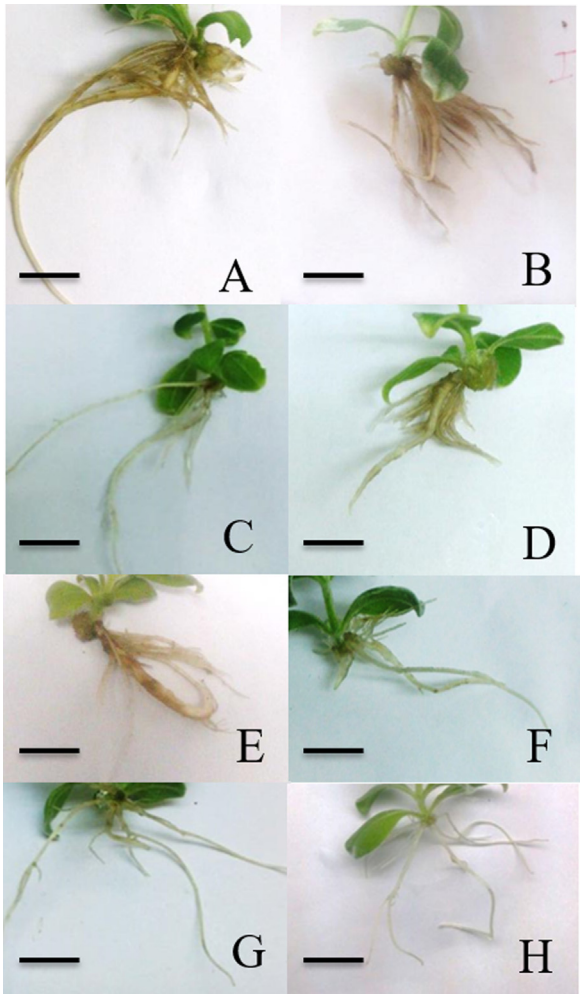

Fig. 4. Rooting of proliferated shoots, 4 weeks after root induction. Development of roots at the base of elongated shoot on MS medium with 12.3 (A), 4.9 (B) and 0.98 (C) μM IBA, 14.3 (D), 5.7 (E) and 1.1 (F) μM IAA and MS (G) and ½ MS (H) medium without hormone (bar = 1 cm).

(22.4%) than upper nodes especially st (0.86%). Maximum number of nodes per shoot was observed in shoots obtained from the shoot tip explants. Without considering the explant type, BAP was better than KN on the shoot proliferation. With increasing the concentration of

Table 4
Essential oil composition of wild and *in vitro* regenerated *Zhumeria majdae*.

No.	Compound	CRI[a]	LRI[b]	Plant material[c]		Identification method[d]
				WP	IVRP	
1	Tricyclene	927	921	0.2	–	RI, MS
2	α-Pinene	938	932	2.0	1.4	RI, MS, CoI
3	Camphene	955	946	4.1	3.5	RI, MS
4	3-Octanone	981	979	2.4	1.1	RI, MS
5	Myrcene	988	988	0.9	0.7	RI, MS, CoI
6	*n*-Octanal	998	998	0.1	–	RI, MS
7	α-Phellandrene	1007	1002	0.1	0.6	RI, MS, CoI
8	α-Terpinene	1019	1014	0.5	0.8	RI, MS
9	Limonene	1032	1024	4.6	8.3	RI, MS, CoI
10	*(E)*-β-Ocimene	1045	1044	0.1	0.3	RI, MS
11	γ-Terpinene	1060	1054	1.3	0.5	RI, MS
12	*cis*-Linalool oxide	1074	1067	0.8	–	RI, MS
13	Terpinolene	1090	1086	1.8	0.1	RI, MS, CoI
14	Linalool	1100	1095	31.7	41.7	RI, MS
15	α-Campholenal	1131	1122	0.21	–	RI, MS
16	Camphor	1160	1141	28.0	32.4	RI, MS
17	Borneol	1174	1165	2.6	0.9	RI, MS, CoI
18	Terpinen-4-ol	1184	1174	2.9	0.2	RI, MS
19	α-Terpineol	1195	1186	3.0	1.5	RI, MS
20	7*Z* -Decenal	1201	1199	0.1	–	RI, MS
21	2,6-Octadiene,1-methoxy-3,7-dimethyl	1222	–	2.6	–	MS
22	Nerol	1231	1227	0.3	0.9	RI, MS
23	Neral	1240	1235	1.2	0.2	RI, MS
24	Geraniol	1252	1249	3.6	–	RI, MS, CoI
25	Geranial	1268	1264	1.1	0.6	RI, MS
26	Isopiperiton	1276	–	0.2	–	MS
27	*Z*-Jasmone	1400	1392	0.2	–	RI, MS
28	*E*-Caryophyllene	1434	1417	1.0	2.7	RI, MS
29	α-Humulene	1468	1452	0.1	0.3	RI, MS
30	Caryophyllene oxide	1600	1582	0.5	–	RI, MS
Monoterpene hydrocarbons				13.8	16.1	
Oxygenated monoterpenes				77.4	78.5	
Sesquiterpene hydrocarbons				1.1	3.0	
Oxygenated sesquiterpenes				0.5	–	
Others[e]				5.4	1.1	
Total identified				**98.2**	**98.7**	

Note:.
[a] CRI, calculated retention indices determined in the present work relative to n-alkanes C6–C24 on DB-5 column.
[b] LRI, literature retention index values (Adams, 2007).
[c] WP, wild plant; IVRP, *in vitro* regenerated plant.
[d] MS, mass spectrum; CoI, co-injection with an authentic sample.
[e] Others, unsaturated and oxygenated hydrocarbons.

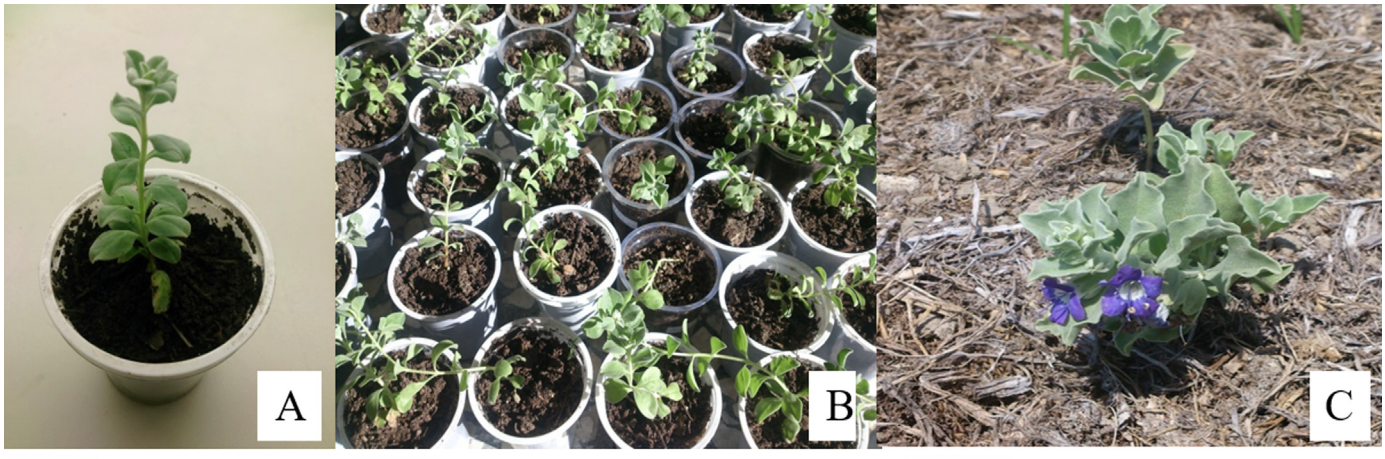

Fig. 5. Acclimatization of regenerated *Zhumeria majdae* plantlets: (A) Transplanted plant in pots after 1 month. Regenerated plantlets of *Z. majdae* after 6 months of acclimatization. Bar = 3 cm.

cytokinins, the number of shoots per explant was increased but the height of plantlets was reduced. The maximum number of shoots was obtained on the medium containing 71.0 μM BAP, while the best shoot proliferation rate was obtained in 17.7 μM BAP.

Maximum number of node per shoot and shoot length was recorded in MS medium containing 2.2 and 4.4 μM BAP, respectively. The highest rooting rate was observed on ½ MS medium hormone-free. Our results strongly suggest that *in vitro*-propagated of *Z. majdae* can be considered as an attractive and alternative source of the plant linalool rich essential oil. Furthermore, the developed protocol appeared to be suitable for sustainable exploitation of plant for cosmetic, perfumery and pharmaceutical use.

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Conflict of interest

The authors declare no conflicts of interest.

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