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Prospects of increasing the presence of *Helianthemum kahiricum* Dell. pastoral North African plant by means of micropropagation

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***Helianthemum kahiricum* has great potential in forage, in traditional medicine, for halting desert encroachment and stabilizing sand dunes through their excellent root systems development, and in the improvement of soil organic matter content. *H. kahiricum* is threatened with extinction because of overgrazing abuse and increasing human disturbance. For these reasons, *in vitro* propagation is essential for developing efficient conservation program. Several cytokinins: indole-3-acetic acid (IAA) ratios and a range of zeatin concentrations were evaluated for their effect on shoot multiplication from apical shoots and nodal segments. The type of cytokinin and the origin of the explants were the most important factors affecting shoot multiplication. The highest shoot multiplication rate was obtained from single-nodal explants on medium hormone free. Increasing zeatin concentrations promotes decreased shoot multiplication independently of explants type, although this effect tends to decrease with higher zeatin concentration. Shoot growth was higher in apical shoots and it was not stimulated by the presence of auxin. A number of experiments were conducted to identify suitable procedures for rooting of *in vitro* produced shoots. Although, rooting frequency rose to 98% by *in vitro* culture on an auxin-free medium, the survival of the plants after 6 months of acclimatization was good (90%).**

Key words: Biotechnology conservation, pastoral species, *ex vitro* rooting, *Helianthemum kahiricum*, *in vitro* culture.

INTRODUCTION

Helianthemum kahiricum Dell. (*H. kahiricum*) is a perennial herb plant [15 cm tall, covered in its various parts by glandular hairs, the leaves are whorled, sessile flowers with five sepals, five yellow petals (Ozenda, 1977), numerous stamens and 3 to 5 carpels defining a unilocular ovary, the fruit is a hairy capsule, photo-synthesis of the plant is C3 belonging to the Cistaceae] (Aïdoud et al., 2006). It is distributed in arid and semi-arid Mediterranean areas. It presents an important ecological, economical and pastoral interest (Radice and Caso, 1990; Hsia and Korban, 1998), besides it has a medicinal interest. Despite its ecological and economic interests, this plant is a rare endemic flora of the western basin of the Mediterranean (Escudero, 2007), as a result of overgrazing. The size of the population is restricted or is greatly diminished; the data indicate that the situation

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Abbreviations: BA, 6-Benzyladenine (cytokinine); **IAA,** indole-3-acetic acid (auxin); **IBA,** indole-3-butyric acid (auxin); **kin,** kinetin (cytokinine); **MS,** Murashige and Skoog medium; **NAA,** 2-naphthalene acetic acid (auxin); **2iP,** 2 isopentenyladenine (cytokinine).

will worsen irreversibly if nothing is done to address this vulnerability. In other words, if the observed situation continues, it is anticipated the disappearance of the specie. The factors responsible include degradation of habitat, exploitation of the species, exposure to pollutants, parasitism, diseases, interspecific competition, climate change, overexploitation; other cause result in the regression in number, but the population level reaches a critical threshold. They have a very important role in the fight against desertification and stabilization of vulnerable areas (Diez et al., 2002). In addition, they are involved in the production of desert truffles (Al-Rahmah, 2001).

Desert truffles, known locally as the "terfess" are edible and wild seasonal mushrooms hypogeal (Mandeed et al., 2007). *H. kahiricum* is considered threatened and is in an extremely precarious situation. The size of their population or their range, or both, is restricted or is greatly diminished. Thus, to maintain the genetic integrity of clones and conservation of this species (Lankova et al., 2001), *in vitro* germination, cultivation microcuttings and stimulation of axillary buds are the most applied in plant micropropagation method (Walali, 1993). For our species, *in vitro* culture seems to be a very interesting alternative for preserving *H. kahiricum* against the scourge of extinction. This work has as purpose the multiplication of the species by micropropagation highlighting the effect of the composition of the medium on the multiplication of plant material.

MATERIALS AND METHODS

Plant material and surface sterilization

Young shoots, 8 to 10 cm length, were collected from 4 to 5-years-old trees of *H. kahiricum* growing in the garden of Arid Lands Institute of Medenine (IRA) (Medenine: latitude 32° 7' 09" N, longitude 11° 38' 26" E, with arid climate characterized by a mean rainfall of 150 mm/year). The types of explants that were used were the nodal and apical explants, defoliated and surface-sterilized according to Romano et al. (1992). Buds were grown under controlled conditions (glasshouse), and developed shoots, 1 to 2 cm in length, were collected for *in vitro* culture according to Romano et al. (1992). These explants were treated with 70% ethanol for 3 min and 15% Clorox (containing 5% sodium hypochlorite) with 0.2% detergent for at least 10 to 20 min for surface sterilization, and then rinsed six times with sterilized distilled water. Explants were individually placed in test tubes (25 to 160 mm) containing 10 ml of MS (Murashige and Skoog, 1962), supplemented with 0.5 mg L^{-1} 6-benzyladenine (BA). Sucrose (2%, w/v) was used as a carbon source and media were solidified with 0.8% (w/v) agar. Media pH was adjusted to 5.8 before autoclaving at 121°C for 20 min. Cultures were incubated at 25 ± 2°C under a 16 h photoperiod at 60 µmol m^{-2} s^{-1} provided by cool-white fluorescent lights for 10 weeks (Iriondi, 1995).

Shoot multiplication

Explants were individually placed in test tubes containing 10 ml of M S (Murashige and Skoog, 1962), supplemented with hormones. During the multiplication stage, many culture media were used: MS with cytokinin, MS with auxin and MS with combination (auxin and cytokinin). Several cytokinins concentrations [zeatin (0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg L^{-1}), 2iP–2-isopentenyladenine (0.5, 1.0 mg L^{-1}), Kin–kinetin and BA (0.5, 1.0 mg L^{-1}) (0.5, 1, 1.5, 2 and 3 mg L^{-1}), and auxins: IAA ratios (0.5, 1, 1.5 mg L^{-1})] were assayed. Twenty (20) cultures per treatment were tested. After 10 weeks, shoot multiplication rate defined as the total number of shoots produced from each initial explants and the length of the longest shoot per culture were evaluated.

Root induction

Shoots were rooted using *in vitro* and *ex vitro* methods, and a combination of both. Individual shoots, 4.0 cm in length, harvested at the end of the multiplication stage after being cultured for 2 weeks on ½ MS growth-regulator-free medium, were used in all rooting assays.

In vitro rooting

For *in vitro* rooting studies, two methods were tested: inclusion of auxins in the rooting medium or dipping the base of the shoots in a concentrated auxin solution. In the first one, shoots were cultured on full or half-strength MS (½ MS), containing IBA or NAA at 1.0 or 2.0 mg L^{-1}. In the second, the basal ends of the shoots were dipped in 1.0 g L^{-1} IBA or NAA for 2 min, followed by culture on MS or ½ MS auxin-free medium. For both methods, shoots were grown in test tubes (450 to 200 mm) containing 20 ml of medium. After 8 weeks of induction rooting frequency, root number and the longest root length per plantlet were evaluated.

Ex vitro rooting

For *ex vitro* rooting, the basal ends of the shoots were dipped in 1.0 g L^{-1} of IBA or NAA for 2 min, potted in peat and perlite (2:1, v/v) and immediately transferred to acclimatization conditions. In another experiment, *in vitro* rooting induction followed by *ex vitro* development of the roots was tried. The shoots were dipped in 1.0 g L^{-1} of IBA or NAA for 2 min, cultured on ½ MS auxin-free medium for 1 week in the dark and then transferred to acclimatization conditions. Rooting was evaluated 8 weeks after induction and was expressed in terms of rooting frequency and the substrate colonization. For all rooting assays (*in vitro* or *ex vitro*), 20 shoots were tested per treatment and the experiment was repeated three times.

Acclimatization

Plantlets with at least five well-developed roots were transferred to plastic pots (150 ml) containing a mixture of peat and perlite (2:1, v/v). Potted plantlets were placed in a growth chamber set at 100% relative humidity, 25 ± 2°C, with a 16 h photoperiod (100 µmol m^{-2} s^{-1}), for three months, then transferred to a glasshouse under natural daylight conditions at 25°C temperature. Plantlets were watered twice a week for 3 months with a diluted solution of ½ MS.

Statistical analysis

The results of analysis of variance (ANOVA) of different parameters were obtained by the software SPSS v.11.5. Multiple comparisons

Table 1. Effects of explants type and cytokinin/IAA balances (mg L^{-1}) on mean number and length of shoots.

Concentration (mg L^{-1})	Number of shoots/culture	Length shoot (cm)
Apical shoots		
0.5 zeatin + 0.5 IAA	3.0^{b}	16.9^{ab}
1.0 zeatin + 1.0 IAA	4.7^{a}	17.1^{ab}
0.5 2iP + 0.5 IAA	2.31^{c}	18.7^{a}
1.0 2iP + 1.0 IAA	2.1^{c}	18.6^{a}
0.5 Kin + 0.5 IAA	2.1^{c}	14.7^{c}
0.5 BA + 0.5 IAA	2.0^{c}	15.09^{bc}
1.0 BA + 1.0 IAA	2.0^{c}	15.6^{bc}
Nodal segments		
0.5 zeatin + 0.5 IAA	3.7^{b}	13.2^{a}
1.0 zeatin + 1.0 IAA	4.5^{a}	13.8^{a}
0.5 2iP + 0.5 IAA	2.7^{cd}	13.0^{a}
1.0 2iP + 1.0 IAA	3.1^{c}	13.5^{a}
0.5 Kin + 0.5 IAA	2.32^{d}	11.07^{b}
0.5 BA + 0.5 IAA	2.4^{d}	15.6^{a}
1.0 BA + 1.0 IAA	2.9^{cd}	7.2^{c}

Means with different letters are significantly different at threshold $p < 0.05$ (Duncan test).

of means and the setting command classes were made by Duncan's test.

RESULTS

The initiation of cultures from axillary and apical segments of *H. kahiricum* taken directly from field material was not possible due to high contaminations (~95%). It decreased after the pre-conditioning treatment of the cuttings. Shoot tip and axillary shoot development from single node explants was observed within 10 to 15 days of culture and all the explants showed growth viability.

Shoot multiplication

The mean number of shoots per explants was significantly affected by cytokines and the origin of explants (Table 1). Nodal shoots allowed a higher proli-feration rate (Table 1) and among the four cytokinins tested in combination with IAA, zeatin gave the best results in terms of mean number of shoots (Table 1). For both apical shoots and nodal segments, best results were observed with 2 mg L^{-1} zeatin with 1 mg l^{-1} IAA. Shoot length was significantly affected by cytokinins, the origin of explants, and their interaction (Table 1). When testing several zeatin concentrations and zeatin: IAA combinations for both explants (apical and nodal shoots), we observed that the mean number and length of shoots were significantly affected by the medium, explants origin and by their interaction (Table 2, Figure 1a and 1b); increasing zeatin concentration promotes shoot multiplication. However, this effect tends to decrease with higher zeatin concentration (2, 2.5 and 3 mg L^{-1}). Shoot growth was higher in nodal shoots than apical shoots and it was not stimulated by the presence of IAA (Table 2). Zeatin and 2iP have been successfully used during shoot proliferation of *H. kahiricum* species (Hsia et al., 1998).

Rooting

The effect of IBA and NAA (1 and 2 mg L^{-1}) added to the culture medium was tested in two macronutrient formulations; full MS and ½ MS. Best results in terms of rooting frequency, mean number of roots and root length were obtained in ½ MS (Table 3). For the two auxins (IBA and NAA) tested, the root length was significantly higher with IBA than NAA. With regard to auxin concentration, 1 mg L^{-1} induced significant root length (Table 3). Basal immersion of shoots in a 1g l^{-1} IBA or NAA solution for 2 min followed by *in vitro* culture on auxin-free medium (Table 3) increased the rate of rooting. Mean root number was significantly affected by the basal medium, auxin, and their interaction. IBA induced a higher number of roots and ½ MS was the most adequate medium. In all the *in vitro* rooting assays, roots developed at the base of the shoots but, most of them grew outside the medium,

Table 2. Effects of explants type and zeatin concentration (mg L^{-1}) combined with IAA on mean number and length of shoots.

Concentration (mg L^{-1})	Number of shoot/explant	Length shoot (mm)
Apical shoot		
0.25 zeatin	1.0^{e}	14.3^{d}
0.5 zeatin	1.51^{de}	15.91^{d}
1.0 zeatin	3.7^{a}	19.02^{a}
1.5 zeatin	2.2^{cd}	18.9^{a}
1.5 zeatin + 0.5 IAA	2.16^{d}	16.3^{b}
2.0 zeatin + 1.0 IAA	2.5^{bc}	15.8^{cd}
2.5 zeatin + 1.0 IAA	3.7^{a}	13.1^{bc}
3.0 zeatin + 1.5 IAA	3.1^{ab}	13.7^{cd}
Nodal segment		
0.25 zeatin	2.7^{d}	11.4^{c}
0.5 zeatin	3.4^{bc}	12.9^{ab}
1.0 zeatin	3.5^{bc}	13.7^{a}
1.5 zeatin	3.6^{c}	13.62^{ab}
1.5 zeatin + 0.5 IAA	2.7^{d}	12.9^{a}
2.0 zeatin + 1.0 IAA	4.18^{a}	11.7^{a}
2.5 zeatin + 1.0 IAA	3.27^{bc}	11.5^{a}
3.0 zeatin + 1.5 IAA	3.9^{b}	9.8^{b}

Means with different letters are significantly different at threshold $p < 0.05$ (Duncan test).

possibly due to poor aeration on the agarified medium. Many shoots rooted were observed either in direct *ex vitro* rooting or *in vitro* rooting induction followed by *ex vitro* development of the roots (Table 4). In both assays, IBA was found to be the auxin determining best substrate colonization. Shoots developed high number of thin roots that spread all over the substrate (Figure 1c). These results are in agreement with previous results with other plants *ex vitro* rooting of micropropagated shoots has been successfully used (Briggs et al., 1994; McCulloch and Briggs, 1994). Furthermore, in commercial practice, micropropagated shoots of plants are rooted *ex vitro* since it is effective.

Acclimatization

The *in vitro* regenerated plantlets were acclimatized in a growth chamber at high relative humidity (90 to 95%) for two months, and then they were transferred to the glasshouse. Results showed that after 6 months of acclimatization, the percentage survival was 60%. These results were improved when shoots were induced to root *ex vitro* with a surviving frequency of 100%. This increase could be due to a more developed and efficient rooting system. Micropropagated plants showed good growth and uniformity *ex vitro* and exhibited normal development. When reintroduced into their natural habitat during eight months, these plants showed 85% of survival. *Ex vitro* rooting was found beneficial both in terms of rooting results (100% rooted shoots) and acclimatization success (100% surviving plants) (Figure 1d and e). The results show the utility of combining *ex vitro* rooting and acclimatization in one step in order to reduce costs of multiplying plants via micropropagation. This micropropagation protocol could be used to clone some of this threatened native plant, and therefore it may represent an important tool for the preservation of biodiversity.

DISCUSSION

Shoot length was significantly affected by cytokinins; the origin of explants and their interaction, present a higher proliferation rate among the four cytokinins tested (zeatin, 2iP–2-isopentenyladenin, Kin-kinetin and BA-6 benzyladenine). The work of Armstrong et al. (2001) on *Ceratopetalum gummiferum* showed that the number of shoots per explants increased with increasing the concentration of BAP, Kinetin, Zeatin and 2iP. However, the use of excessive concentrations cytokinin causes a decrease in the number of shoots per explants, the shoot

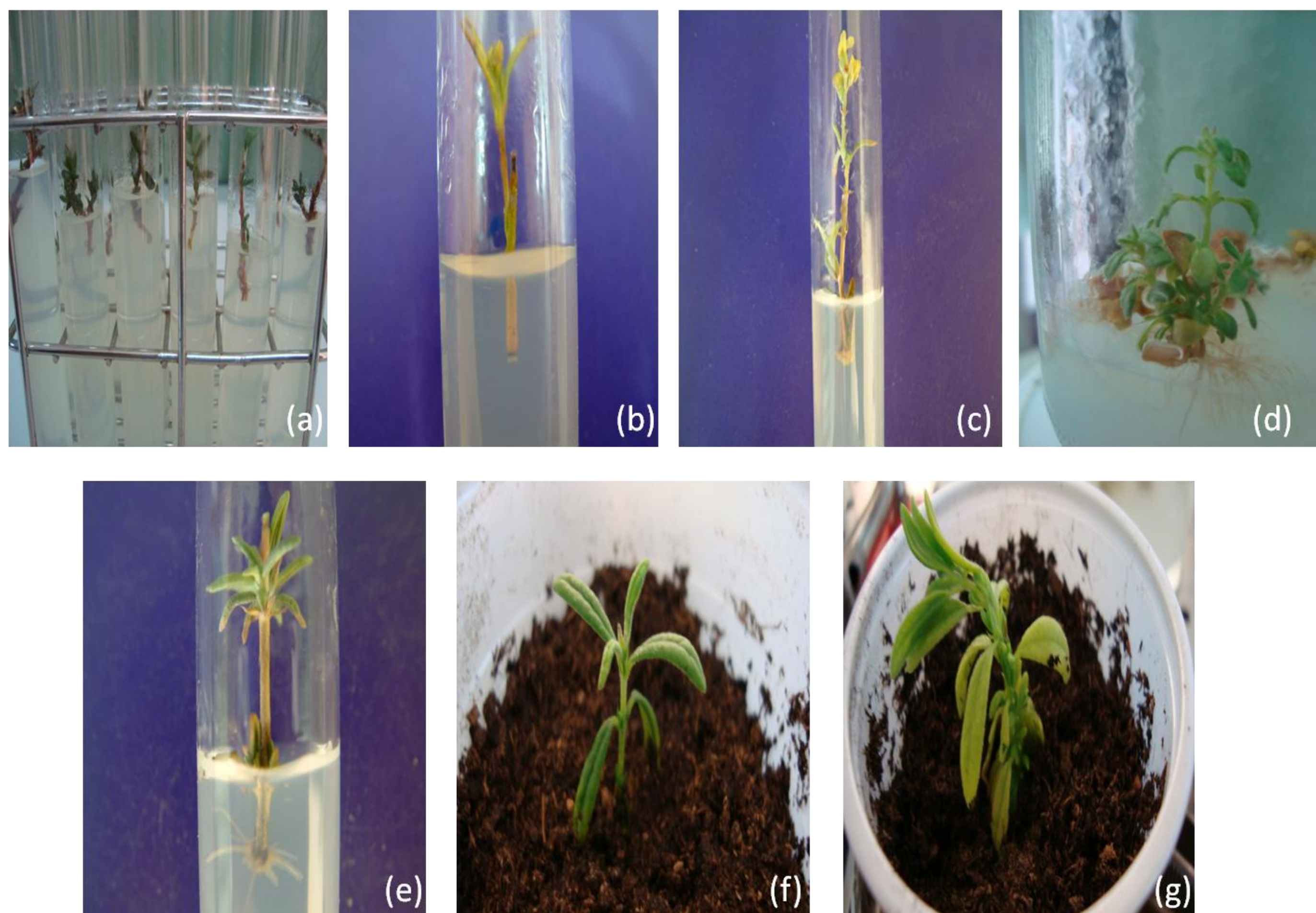

Figure 1. (a) Shoots at the beginning of multiplication phase in medium containing 1.0 mg L^{-1} zeatin. **(b)** Proliferation of axillary shoots from nodal segment on MS medium supplemented with 1.0 mg L^{-1} zeatin. **(c)** Proliferation of apical shoot on MS medium supplemented with 1.0 mg L^{-1} zeatin, (d) plant regenerated from nodal segment with 3 shoots. **(e)** Root regenerated from shoot cultured on MS medium containing 1.0 mg L^{-1} ANA, (f) *in vitro* regenerated planted of *Helianthemum kahiricum* transferred into plastic cup. **(g)** Acclimatized plant transplanted soil in the glasshouse conditions.

length and shoots weight. Shoot growth was higher in nodal segments that the apical shoots and it was not stimulated by the presence of IAA. The addition of auxin has a negative effect on the induction of shoots. Figueiredo et al. (2001) have shown that increasing the concentration of NAA decreases the proliferation of axillary shoots *Rollina mucosa*. The effect of auxin-cytokinin combination was also studied by Fracaro et al. (2001) for *Cunila galioides*. These studies show that the addition of different auxins: NAA, IAA or IBA to a multiplication medium (MS + BAP) significantly reduced the number of shoots per explants. However, Souayah et al. (2003) showed that in *A. halimus*, adding NAA enhances the rate of multiplication but with increased callus. The addition of BAP in *H. lippii*, improves the rate of buds, but the increase in the concentration of BAP induced a decrease in growth of shoots. Best results in terms of rooting frequency, mean number of roots and root length were obtained in ½ MS (Table 3).

Morte and Honrubia (1992) showed that the roots of *Helianthemun almeriense* occur after dilution of the culture medium. Similarly, for *Atriplex halimus* (Souayah et al., 2003), for which rooting is obtained on media without growth regulators indeed increased with diluting the mineral medium.

Conclusion

The shoot growth of *H. kahiricum* was higher in nodal shoots than apical shoots and it was not stimulated by the presence of IAA, shoot length was significantly

Table 3. Effect of media (MS and ½ MS), auxins (NAA and IBA) added to the medium and concentration (1 and 2 mg L^{-1}) on rooting frequency, mean number and length of root.

Medium	Auxin	Concentration (mg L^{-1})	Rooting (%)	Number of roots/explants	Length root (mm)
***In vitro* rooting**					
MS	NAA	1	61	5.4^{a}	10.7^{b}
		2	64	3.1^{b}	8.0^{b}
	IBA	1	42	5.2^{a}	17.4^{a}
		2	23	4.9^{a}	14.3^{a}
½ MS	NAA	1	69	6.8^{a}	17.0^{cd}
		2	83	6.9^{a}	14^{d}
	IBA	1	80	6.4^{a}	27.9^{a}
		2	82	5.8^{a}	19.4^{b}
***Ex vitro* rooting**[a]					
MS	NAA	1	94	7.8^{b}	6.9^{c}
	IBA	1	91	7.6^{b}	8.1^{b}
½ MS	NAA	1	94	7.6^{b}	7.2^{c}
	IBA	1	98	11.7^{a}	12.6^{a}

Means with different letters are significantly different at threshold $p < 0.05$ (Duncan test). [a]The basal ends of the shoots were dipped in 1 g l^{-1} auxin for 2 min, followed by culturing on auxin-free medium.

Table 4. Influence of auxins (IBA and NAA, 1 g L^{-1}) applied by basal immersion of the micropropagated shoots (during 2 min) on rooting frequency and substrate colonization.

Treatment	Rooting (%)	Substrate colonization (%)
***Ex vitro*[a]**		
NAA	100	64^{b}
IBA	100	76^{a}
***In vitro/ex vitro*[b]**		
NAA	100	62^{b}
IBA	100	73^{a}

Means with different letters are significantly different at threshold $p < 0.05$ (Duncan test). [a] Basal ends of the shoots were dipped in 1 g L^{-1} auxin for 2 min, potted in peat and perlite (2:1, v/v) and immediately transferred to acclimatization conditions; [b] Shoots after the basal immersion described earlier were cultured on ½ MS auxin-free for 1 week in the dark and then transferred to acclimatization conditions.

affected by cytokinins. The best results in terms of rooting frequency mean number of roots and root length were obtained in ½ MS. For the two auxins (IBA and NAA) tested, the longest root length was significantly higher with IBA than NAA. These results were improved when shoots were induced to root *ex vitro.* Results showed that after 6 months of acclimatization, the percentage survival was 60%. Micropropagated plants showed good growth and uniformity and exhibited normal development. When reintroduced into their natural habitat during 8 months, these plants showed 85% of survival.

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