

Full Length Research Paper

Micropropagation of *Helianthemum lippii* L. var *Sessiliflorum* (Cistaceae) an important pastoral plant of North African arid areas

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Helianthemum lippii L. is a perennial, little brush distributed in sandy regions of southern Tunisia as well as of Mediterranean basin. It presents an important ecological, economical and pastoral interest. All these characteristics encourage us to try the *in vitro* propagation because it is a feasible alternative for the rapid multiplication and the preservation of plants. A very high frequency of sprouting and shoot differentiation were observed in the primary cultures of nodal explants of *H. lippii* on MS medium, without growth regulators or with a lower concentration of 6-benzylaminopurine (BAP, 0.5 mg L⁻¹ or 1.0 mg L⁻¹ BAP). *In vitro* proliferated shoots were multiplied rapidly by culture of shoot tips on MS medium free or with BAP (0.5 to 2.0 mg L⁻¹) which produced the greatest multiple shoot formation. BAP had a positive effect on multiplication and on growth, but a concentration that exceeds 2.0 mg L⁻¹ decreased the growth. A high frequency of rooting (71%) with development of healthy roots was observed with shoots cultured on MS/8 medium hormone-free. After *in vitro* rooting, and transfer to soil, a number of plantlets suitable for reintroduction in nature were produced.

Key words: *Helianthemum lippii*, axillary buds, micropropagation, plant conservation.

INTRODUCTION

Helianthemum lippii L. (*H. lippii*) is a perennial little brush, which occurs on sandy soils and sandy limestone gypsum low-lying in North African (Raynaud, 1987; Escudero et al., 2007). It is distributed in the bioclimatic semi-arid (Perez-Garcia and Gonzalez-Benito, 2006; Belghith, 2007) where it is well suited to severe climatic conditions in Southern Tunisia. It presents an important ecological, economical and pastoral interest and plays a pivotal role of struggle against desertification and the stabilization of vulnerable sites (Diez et al., 2002). Besides, it has a medicinal interest because the powder or the compress of the aerial part is used to treat

coetaneous lesion. This plant is known to be usually associated to desert truffles. *H. lippii* is the host plant for several species of desert truffles, which have interest for food, medicinal and economic purposes, development of the rural and local populations, (Plenchette and Duponnois, 2004; Slama et al., 2006; Mandeel et al., 2007), and in particular *Terfezia boudieri* Chatin and *Tirmania nivea* (Desf) (Slama et al., 2006). The presence of *H. lippii* is in a continuous regression. This plant is highly affected by the change in their floristic composition under the effect of overgrazing, land clearing and increased pastoral care (Aidoud et al., 2006). *H. lippii*

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Abbreviations: BAP, 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).

becomes endangered, rare and endemic flora of the western basin of the Mediterranean Sea (Escudero, 2007). All these characteristics encourage us to try the *in vitro* propagation. *In vitro* culture seems to be a very interesting alternative to preserve *H. lippii* against the scourge of extinction under effect of overgrazing, land clearing and increased pastoral care. This work is the multiplication of the species and this was through the micropropagation by axillary budding.

MATERIALS AND METHODS

Plant material

Young shoots were collected from 4 to 5-years-old trees of *H. lippii* growing in the garden of Arid Lands Institute of Medenine (IRA) (Medenine: latitude 32° 57' 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 explants (1 cm) from bud to ensure multiplication by axillary budding.

Disinfection

Stem cuttings, 8 to 10 cm length, were collected from plants growing in southern Tunisia (Medenine: latitude 32° 57' 09" N, longitude 11° 38' 26" E, with arid climate characterized by a mean rainfall of 150 mm/year), defoliated and surface-sterilized and washed with fungicide (Benlate 1 g/l) for 20 min to remove all surface adherents and then left in running tap water for 10 min. Subsequently, the defoliated twigs were surface decontaminated with 0.01% (w/v) mercuric chloride for 15 min. The treated twigs were washed several times with sterile distilled water and finally the use of a solution of sodium hypochlorite (NaOCl 50%) at different immersion times (5 to 20 min). Prior to inoculation, the twigs were trimmed into 1-cm-long pieces each having one or two nodes.

Micropropagation of *Helianthemum lippii*

Initiation

Explants were individually placed in test tubes (25 to 160 mm) containing 10 ml of MS (Murashige and Skoog 1962), supplemented with hormones. The hormones used were 6-benzylaminopurine (BAP: 0.25 to 2.0 mg L⁻¹) and α -naphthalene acetic acid (NAA: 0.5 and 1.0 mg L⁻¹). 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 μ mol m⁻² s⁻¹ provided by cool-white fluorescent lights for 10 weeks (Iriondi, 1995).

Multiplication

Shoots (1 ± 0.3 cm) obtained from the first step of micropropagation were transferred to MS medium with different concentrations of BAP added (0.25 to 2.0 mg L⁻¹).

Rooting

The root induction was performed on MS media diluted (MS/2, MS/4, MS/6 and MS/8) and MS medium containing different concentrations of acetic acid α -naphthalene (NAA: 0.25 to 1.5 mg L⁻¹

¹). All cultures were maintained in growth room for 3 months at 25 ± 2°C under a photoperiod (16/8 h) at a photosynthetic flux of 12, and 6 μ mol m⁻² s⁻¹ provided by cool daylight fluorescent lamps.

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 μ mol m⁻² s⁻¹), for three months, and 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 1/2MS.

Statistic analysis

The response data was arcsine transformed before analysis. All data were subjected to one-way analysis of variance (ANOVA). For each treatment, 20 explants were cultured.

RESULTS

Disinfection of explants

The appropriate protocol of disinfection is to soaking explants in mercuric chloride (0,01%) for 15 min, then immersion in bleach (NaOCl 50%) for 20 min; this protocol gives a low rate of contamination. The treatment of explants with the solution of HgCl₂ (1 g L⁻¹) for 20 min gave satisfactory results. In fact, this treatment ensures complete sterilization without damaging the explants compared to 10 and 15 min soaking. In the literature, several types of disinfectants are used for the sterilization of plant material such as alcohol, petroleum ether, calcium hypochlorite, sodium hypochlorite, acetone, etc at concentrations and durations of dips that vary depending on the species (Phatak and Heble, 2002; Moran et al., 2003; Tan et al., 2004).

Induction of auxiliary bud

The effect of the concentration and nature of growth regulators on bud explants is reported in Table 1. In the absence of growth regulators, the rate of axillary bud was very high (almost 100%) than it was for a complete basal medium (MS) or in medium supplemented with 0.5 or 1.0 mg L⁻¹ of BAP (M₂ and M₃). The addition of a cytokinin (BAP); however, favored the development of axillary buds.

Explants that have ridden the first giving a percentage of bud highest (~100%) are those that have been grown in a medium supplemented with 0.5 mg L⁻¹ of BAP. The combination of BAP and NAA at a dose of 0.25 mg L⁻¹ was accompanied by a decrease in the percentage of bud break (30%) and a decrease in shoot length (0.56 cm) (Figure 1a, b). Endogenous hormones of tissue *H. lippii* are able alone to stimulate axillary bud.

Table 1. Effect of growth regulators on induction of axillary bud tissues *Helianthemum lippii* L.

Medium	Composition (mg.L ⁻¹)	Bud (%)	Means long shoots (cm)
M ₁	MS	100	1.08 ^c
M ₂	MS + 0.5 BAP	100	2.28 ^a
M ₃	MS + 1.0 BAP	100	1.2 ^b
M ₄	MS + 2.0 BAP	60.0	0.58 ^d
M ₅	MS + 1.0 BAP + 0.25 NAA	30.0	0.56 ^d
M ₆	MS + 1.0 BAP + 0.5 NAA	27.4	0.8 ^{bc}
M ₇	MS + 1.0 BAP + 1.0 NAA	21.7	0.58 ^d

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

The addition of growth substances also increased to an excessive concentration of growth regulators making them toxic.

The combination of 1.0 mg L⁻¹ of BAP and 0.25 mg L⁻¹ NAA (M₅) however positively influenced callus thus the addition of NAA to the culture medium promotes callus formation at the expense of bud development. This can be explained by the fact that the addition of NAA in the culture medium decreased bud development at the expense of callus (Figure 1c). At a higher dose of NAA (1.0 mg L⁻¹) (M₇), the percentage of bud and shoot length decreased (21%, 0.58 cm). Also, the leaves begin to wrap and the shoots begin to be less vigorous. However, other authors such as Figueiredo et al. (2001) showed that auxin-cytokinin combination decreased the proliferation of shoots in *Periploca laevigata* and *Rollinia mucosa* respectively.

Proliferation of axillary shoots

The results (Table 2) show that the rate of axillary bud varied between 60 and 90%. Indeed, in all walks of multiplication, bud occurred after two weeks of culture; whereas it is three weeks for primary buds. This result can be explained by the fact that explants from the establishment phase of culture have acquired juvenile characters that can be considered as a response to *in vitro* culture.

The results showed that the rate of increase is positively correlated with the concentration of BAP. But arriving at a concentration of 2 mg L⁻¹ of BAP (M₆), the number of nodes/shoot decreases and the BAP beginning to have an inhibitory effect; this is justified by the low percentage of multiplication as well as the loss of shoot vigor (Figure 1c, d).

The effect of cytokinins on axillary buds has been studied by several authors. Thus, Souayah et al. (2003) found that the addition of BAP improves the rate of bud in *Atriplex*, but the increase in the concentration of BAP induced a decrease in the number of axillary shoots strong and no vitrified.

Rooting

Adventitious root formation is a phenomenon of great importance for the success of propagation. This is very difficult to achieve in wood. Rooting was obtained on media without growth regulators. The rate of rooting and number of roots per explants differ according to the environment (medium). On media supplemented with different concentrations of auxin (NAA), no evidence of rooting was observed. The findings highlight the appearance of callus at the base of the shoots. The rooting rate was from 32 to 71.8% for MS to MS/8. Rooting was observed after 3 to 4 weeks of culture. After this period, there was a gradual increase in the number of roots. Concerning the effect of dilution of minerals on rooting, we noted that the media MS/4, MS/6 and MS/8 gave the best rooting rate (54.2, 66.06 and 71.8%, respectively). This factor clearly affects the rate and number of roots/explant which considerably increased with the dilution of mineral medium (Figure 1f).

In examining these results, we noted that rooting was obtained on media without growth regulators. Uploading of auxin in the culture medium strongly inhibits rooting. This is due to the wealth of *H. lippii* in endogenous growth regulators. Rooting was obtained on media without growth regulators, on media supplemented with different concentrations of auxin (NAA), no evidence of rooting was observed in *H. lippii* (Table 3).

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 show that after six months of acclimatization, the percentage survival was 60%. 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 8 months, these plants showed 45% of survival (Figure 1f, h).

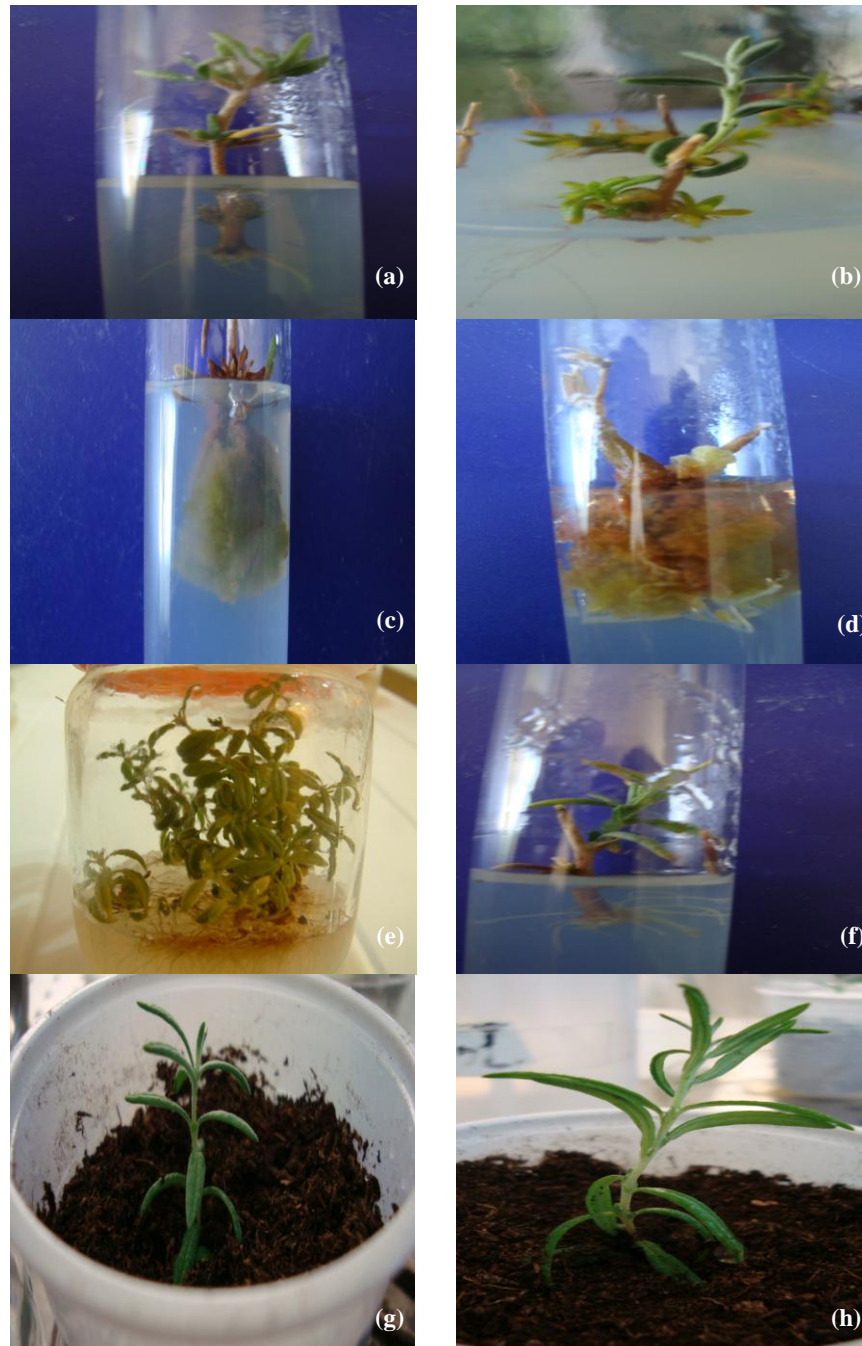


Figure 1. Proliferation of auxillary shoots of *Helianthemum lippii* on (a) MS + 1.0 mg.L⁻¹BAP + 0.25 mg.L⁻¹ NAA; (b) MS medium; (c) Callus induction from nodal segment on MS + 0.50 mg.L⁻¹ BAP + 0.25 NAA mg.L⁻¹; (d) MS + 1.0 mg.L⁻¹ BAP + 1.0 NAA mg.L⁻¹; (e) MS + 1.0 mg.L⁻¹ BAP; (f) F, rooting on MS/8; (g + h) regenerated plantlets transferred in micropots (100%).

DISCUSSION

Explants of *H. lippii* presents a good disinfection in mercuric chloride solution (1%) after immersion in bleach (50%) for 20 min; some plants such as *Carissa carandas*

have been disinfected by dichloride of mercury (HgCl₂) at different concentrations and different soaking time. Other species such as *Cyrtontus* were sterilized by hypochlorite of sodium (NaOCl) (Moran et al., 2003). The addition of a cytokinin with low concentrations (BAP: 0.5 to 1.0 mg L⁻¹),

Table 2. Effect of BAP concentration on the multiplication phase of *Helianthemum lippii* vitro pousses.

Medium	Composition (mg.L ⁻¹)	Bud (%)	Means fibre of nodes/shoot	Means long shoots (cm)
M ₁	MS + 0.25 BAP	68.6	2.4 ^{ab}	1.32 ^d
M ₂	MS + 0.5 BAP	80	3.6 ^a	2.01 ^c
M ₃	MS + 0.75 BAP	60	3.46 ^b	2.20 ^b
M ₄	MS + 1.0 BAP	90	3.4 ^b	2.88 ^a
M ₅	MS + 1.5 BAP	80	3b ^c	2.98 ^a
M ₆	MS + 2.0 BAP	80	1.8 ^d	2.36 ^b

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

Table 3. Effect of dilution and NAA (auxin) on the induction of rooting vitro pousses of *Helianthemum lippii*.

Medium	Composition (mg.L ⁻¹)	Rooting rate (%)	Number of roots/explant	Long means roots (cm)
M ₁	MS	32 ^e	5,6 ^c	1.85 ^b
M ₂	MS/2	48.6 ^d	5.6 ^c	2 ^c
M ₃	MS/4	54.7 ^c	6.6 ^b	2.4 ^d
M ₄	MS/6	66.06 ^b	7.8 ^a	3.08 ^e
M ₅	MS/8	71.80 ^a	7.4 ^a	4.48 ^f
M ₆	MS + 0.25 NAA	0	0	0
M ₇	MS + 0.5 NAA	0	0	0
M ₈	MS + 1 NAA	0	0	0
M ₉	MS + 1.5 NAA	0	0	0

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

avored the development of auxiliary buds; however, the increase in concentration had a negative effect. The work of Armstrong et al. (2001) on *Ceratopetalum gummiferum* showed that the number of shoots per explants increased with increasing concentration of BAP, Kinetin, Zeatin and 2iP. However, the use of excessive concentrations cytokinin caused a decrease in the number of shoots per explant, the shoot length and shoots weight. These doses also cause browning of explants bases, yellowing shoots, deformation and stunting of the leaves. The addition of auxin had a negative effect on the induction of auxiliary bud. Figueiredo et al. (2001) have shown that increasing the concentration of NAA decreases the proliferation of axillary shoots *R. 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. The toxic effect of high concentrations of growth regulators was observed when explants of hypocotyl and epicotyl of *R. mucosa* are

grown in culture media containing high doses of kinetin, BAP or 2,4-D (Figueiredo et al., 2001). Similarly, Zhijun and al. (2001) explained the toxic effect of the use of high concentrations of BAP for axillary bud by the fact that the addition of exogenous BAP causes an imbalance in the concentration of growth regulators causing toxicity to cultured buds. The rooting of shoots in culture medium without growth regulators was also obtained for *Vaccinium macrocarpon* (Debnath et al., 2001). Similarly, Iriondo et al. (1995) working on *Helianthemum polygonoides* showed that the rooting of this species can be done even on MS media without auxin. Also, Morte and Honrubia (1992) showed that the roots of *Helianthemum 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

In vitro propagation is a feasible alternative for the rapid multiplication and the preservation of germoplasm. With the development of biotechnology and its contribution axis at all levels (genetic resources conservation, sanita-

tion plants, creating new genetic combinations, etc.), *in vitro* propagation methods are essential for plant genetic resources management and are becoming increasingly important for conservation of rare and endemic plant species (Sudha et al., 1998; Iankova et al., 2001). Similarly, these techniques facilitate the application of genetic manipulation procedures and long-term storage (Hawkes et al., 2000). Micropropagation of *H. lippii* was successfully implemented using the explants from specimens of the Medenine region (Tunisia, IRA plot: Institute of Arid Regions). To obtain this result, it was necessary to develop an adequate disinfection system in order to have sterile cultures. This disinfection protocol requires the use of a fungicide and then of a solution of mercuric chloride (1 g L^{-1}) for a period of 20 to 30 min. The rate of axillary bud is almost 100% on MS or MS supplemented with low concentration of BAP (does not exceed 0.5 mg L^{-1} of BAP), while the combination of BAP and NAA caused a decrease in the percentage of bud and shoot length growth.

Results show that the rate of multiplication is positively correlated with the concentration of BAP, at a high concentration; the BAP has an inhibitory effect; this is justified by the low percentage of multiplication as well as the loss of shoot vigor and decreases of number nodes per shoot. Rooting was obtained on media without growth regulators. The rate of rooting and number of roots per shoot differ according to the medium composition minerals. On media supplemented with different concentrations of auxin (NAA), no evidence of rooting was observed. Endogenous hormones of tissue *H. lippii* are able alone to stimulate axillary bud. The addition of growth substances also increased to an excessive concentration of growth regulators making them toxic.

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