

## ***In vitro* Micropropagation of *Helianthemum lippii* L. var *Sessiliflorum* (Cistaceae): A Valuable Pastoral Plant**

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**Abstract:** A rapid micropropagation protocol was developed for *Helianthemum lippii* using nodal explants derived from mature trees. Multiple shoots were induced directly from the axis of nodal explants on MS medium containing 30 g l<sup>-1</sup> sucrose, 8 g l<sup>-1</sup> agar and different concentration of BAP (0.25, 0.5, 0.75, 1, 1.5 and 2 mg l<sup>-1</sup>). The results showed that MS medium supplemented with 1.5 mg l<sup>-1</sup> BAP gave the best caulogenic response with an average of 0.98 shoots per explants developed on 95% of the cultures, after 4 weeks. *In vitro* regeneration shoots developed roots directly from the basal cut ends of shoots without an intervening callus phase, either on MS alone or MS medium supplemented with 1 mg l<sup>-1</sup> indol butyrique acid (IBA) after 30 days of incubation. More than 90% of the rooted survived in the greenhouse, after 3 months of transfer. These plantlets are growing well without any phenotypic aberrations.

**Key words:** Cistaceae • Micropropagation • *Helianthemum lippii* • Culture *in vitro*

### **INTRODUCTION**

*Helianthemum lippii* L. is an herbaceous perennial plant (15cm tall) belonging to the Cistaceae [1, 2]. It's distributed in arid and semi-arid areas in the Mediterranean. It presents an important ecological, economical and pastoral interest and plays an important role of struggle against desertification and the stabilization of vulnerable sites [3]. Besides it has a medicinal interest. The powder or the compress of the aerial part are used to treat cutaneous lesion. It intervenes also in the production of desert truffles, locally knowed "terfess" [4]. These mushrooms grown in symbiotic association with roots of host plants (cistaceae) especially *Helianthemum lippii*. All these characteristics encourage us to its *in vitro* propagation. *In vitro* propagation is a feasible alternative for the rapid multiplication and the preservation of germoplasm. *In vitro* propagation ensures the production of true-to-type plants in limited space and time. The propagation

from elite mature plants is preferred for this purpose as they are selected on past performances. This communication reports an efficient protocol for regeneration of plants of *H.lippii* using nodes derived from mature trees.

### **MATERIALS AND METHODS**

Young shoots were collected from 4-to-5-years-old trees of *Helianthemum lippii* growing in the garden of Arid Lands Institute of Medenine (IRA). The shoots were defoliated and washed with fungicide (Benlate 1g.l<sup>-1</sup>) 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. Prior to inoculation, the twigs were trimmed into 1-cm-long pieces each having one or two nodes.

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The nodal explants (1cm) were implanted into MS medium [5] supplemented with 8 g l<sup>-1</sup> agar and 20 g l<sup>-1</sup> sucrose. The media were supplemented with different concentrations (0, 0.25, 0.5, 1, 1.5, 2 mg l<sup>-1</sup>) of BAP or (0, 0.25, 0.5, 1, 1.5 mg l<sup>-1</sup>) kinetin (Sigma-Aldrich). The pH of the media was adjusted to 5.8 with 1 N HCl or 0.1 N NaoH, before addition of agar. The tubes containing 10 ml of media were closed with metallic caps. Media were autoclaved at 1.05 Kpa at 121°C for 20 min. All cultures were maintained at 25±2°C under a 16 h photoperiod at a photosynthetic flux of 12.6 μmol m<sup>-2</sup> s<sup>-1</sup>, provided by cool daylight fluorescent lamps.

For rooting of *in vitro* regenerated shoots, the shoots were either implanted on MS medium alone or MS with different concentrations (0.25, 0.5, 1, 1.5 mg l<sup>-1</sup>) of IBA.

After 4- to 5-weeks, rooted shoots were removed from the culture tubes and washed with water before being transferred to small pots containing garden soil/vermiculite (1:1) and kept in a mist house. After acclimatization in the mist house for 3 months, they were transferred to a greenhouse.

The culture responses were expressed in terms explants sprouting percentage, number of regenerants (shoots or roots) per explant, average length of shoots or roots. A completely randomized block design with three replications was used. The response data was arcsine transformed before analysis. All data were subjected to one-way analysis of variance (ANOVA). Fisher's least significant differences (LSD) at the 5% level were used to analyse the differences between the means. For each treatment of a repiquage experiment, 24 explants were cultured.

## RESULTS AND DISCUSSION

The initial cultures of the explants derived from the mature trees. The offshoots collected during the months of March to April elicited the best morphogenic response.

After successful decontamination of cultures, the other major constraint was the slow growth and low percentage of morphogenic response. On MS basal medium, the dormant axillary buds proliferated to shoots after 2 weeks of culture in 60% of the cultures. The dormant buds became visible after 15 days of incubation. The supplementation of MS medium with different concentrations (0, 0.5, 1, 1.5, 2 mg l<sup>-1</sup>) of BAP enhanced the morphogenic potential of explants. Addition of BAP to the medium not only enhanced the multiplication rate but also favored the proliferation of healthier shoots. At 1 mg l<sup>-1</sup> BAP, 100% of the cultures developed an average 1.3 shoots per explants. Owing to the low caulogenic response from the nodal explants derived from field-grown plants, further experiments were carried out using the microcuttings derived from the shoots regenerated from the micropropagated cultures.

To maximize the shoots proliferation capacity of the nodal explants derived from the microshoots, different concentrations (0, 0.5, 0.75, 1, 1.5, 2 mg l<sup>-1</sup>) of BAP were applied. BAP facilitated the development of healthy shoots. All the used concentrations of BAP produced multiple shoots in more than 94% of the explants (Table 1, Fig. 1A). However, 0.5 mg l<sup>-1</sup> proved to be optimal, producing an average of 2.7 shoots per explants with an average shoot length of 2.28 cm (Table 1, Fig. 1A). The same observation was indicated by Souayah *et al.* [6] for *Atriplex halimus* under low BAP concentration.

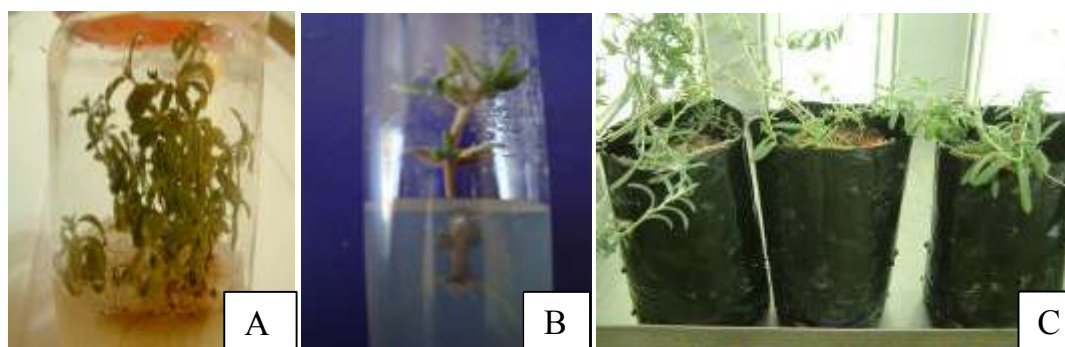


Fig.1: *In vitro* regeneration of nodal explants derived from mature tree of *H. lippii*. (A) Nodal explants with multiple shoot development on MS medium supplemented with 0.5 mg l<sup>-1</sup> BAP after 30 days of culture. (B) Stunted and malformed shoots developed on MS medium supplemented with 0.5 mg l<sup>-1</sup> kin after 30 days of culture. (C) Development of roots from the base of an *in vitro* regenerated shoot on MS medium supplemented with 0.25 mg l<sup>-1</sup> IBA after 21 days. (D) Three-month-old regenerated plants in the greenhouse.

Table 1: Percentage response of nodal explants of *H.lippii* to shoot cultured on MS medium supplemented with different concentrations of BAP, after 30 days of culture.

BAP (mg l <sup>-1</sup> )	Percentage response	Number of shoots/ Explants	Average shoot length (cm)
0	100	3.9 <sup>a</sup>	1.08 <sup>ab</sup>
0.5	100	2.7 <sup>a</sup>	2.28 <sup>c</sup>
0.75	70	1.2 <sup>a</sup>	1.32 <sup>b</sup>
1	100	1.3 <sup>a</sup>	1.2 <sup>b</sup>
1.5	80	2 <sup>a</sup>	0.98 <sup>ab</sup>
2	60	1.2 <sup>a</sup>	0.88 <sup>a</sup>

Values followed by the same letter in each column are not significantly different ( $p \leq 0.05$ ).

Table 2: Percentage response of regenerated shoots of *H. lippii* to root cultured on MS medium supplemented with different concentrations of ANA, after 30 days of culture.

ANA (mg l <sup>-1</sup> )	Percentage response	Number of roots/ explant	Average root length (cm)
0	52 <sup>a</sup>	5.6 <sup>a</sup>	5.8 <sup>a</sup>
0.25	33 <sup>b</sup>	3.47 <sup>b</sup>	5.1 <sup>a</sup>
0.5	19 <sup>c</sup>	2.19 <sup>c</sup>	4.3 <sup>b</sup>
1	17 <sup>bc</sup>	1.07 <sup>bc</sup>	3.7 <sup>c</sup>
1.5	11 <sup>d</sup>	1 <sup>d</sup>	3.3 <sup>bc</sup>

Values followed by the same letter in each column are not significantly different ( $p \leq 0.05$ ).

At higher levels of BAP, though there was no significant difference ( $p \leq 0.05$ ) in percentage of explants developing shoots and the average number of shoots per explants decreased significantly ( $p \leq 0.05$ ). The differential caulogenic responses of cytokinins have been documented previously for *Rollin mucosa* [7]. To further proliferate cultures, shoots were subcultured every 4 weeks on fresh medium. This mode of multiplication ensured continuous supply of shoots for a longer period of time without any evidence of decline in the morphogenic potential.

Three-to four-cm-long regenerated shoots were transferred to rooting medium. On the MS basal medium the induction of roots took place in 52% of the cultures after 4 weeks. These roots were very strong. To attenuate the rooting response, MS medium was further supplemented with various concentrations (0.25, 0.5, 1 or 1.5 mg l<sup>-1</sup>) of ANA. On IBA-supplemented medium, at all concentrations, root development was accompanied with callus formation. However, fragile root systems were obtained on the ANA-supplemented medium, with 0 mg l<sup>-1</sup> concentration exhibiting the best response (Table 2). At this concentration 33% shoots developed roots with an average of 3.47 roots per shoots. At higher concentrations of ANA both percentage of shoots forming roots as well as the number of roots decreased

significantly ( $p \leq 0.05$ ). Auxins are widely used for induction of roots on regenerated shoots. However, not all auxins are equally effective for the purpose and their response varies significantly with the plant used [8].

After 5-6 weeks of root induction, the regenerated plantlets were washed several times with water to remove all adhering culture medium and then planted in 10 cm pots containing a mixture of garden soil and vermiculite (1:1). The transferred plantlets were initially kept in the mist house for 3-4 weeks, until they started to developed new leaves. Of the transferred plants 86% plantlets survived in the mist house (Fig. 1C). After 1 month of incubation in the mist house, the pots were transferred to the greenhouse where more than 90% of the plantlets are surviving (Fig. 1D). The *in vitro* regenerated plants grew well in the greenhouse without any phenotypic aberrations.

Efficient regeneration protocols for *H. lippii* have been established. All the plants transferred to the greenhouse showed a high homogeneity without obvious morphological evidence of somaclonal variation. Micropropagation would ensure a continuous supply of plants in limited time and space, thereby ruling out the dependence on natural stands to fulfill the growing demands for the plant material.

#### ACKNOWLEDGEMENT

The authors acknowledge the technical assistance rendered by all members of Range Ecology Laboratory in Arid Land Institute Tunisia; Especially TLIG Abdelmajid, LOUHICHI Marwen, SEKREFI Mansour and YAHIA Boutheina.

#### REFERENCES

- Escudero, A., I. Marti, A. Nez, Cruz, M.A.G. Ota'lor and F.T. Maestre, 2007. Soil lichens have species-specific effects on the seedling emergence of three gypsophile plant species. *J. Arid Environments.*, 70: 18-28.
- Aïdoud, A., E. Le Floc'h and H.N. Le Houérou, 2006. Les steppes arides du nord de l'Afrique. *Sécheresse*. 17-1(2): 19-30.
- Diez, J., J.L. Manjon and F. Martin, 2002. Molecular phylogeny of mycorrhizal desert truffles (*Terfezia* and *Tirmania*), host specificity and edaphic tolerance *Mycologia*, 94: 247-259.

4. Mandeel, Q.A. and A.A.A. Al-Laith, 2007. Ethnomycological aspects of the desert truffle among native Bahraini and non-Bahraini peoples of the Kingdom of Bahrain. *J. ethnopharmacol.*, 110: 118-129.
5. Murachige, T. and F. Skoog, 1962. A revised medium for rapid growth and biossays with tobacco tissue culture. *Physiologia Plantarum*, 15: 473-497.
6. Souayah, N., M.L. Khouja, M.N. Rejeb and S. Bouzid, 2003. Micropropagation d'un arbuste sylvo-pastoral, *Atriplex halimus* L. (Chénopodiacées). *Cahiers Options Méditerranéenne*.
7. Figueiredo, S., N. Albarello and V. Viana, 2001. Micropropagation of *Rollinia mucosa* (Jacq.). *In vitro Cell. Dev. Biol.-Plant*, 37: 471-475.
8. Swamy, S.L., S. Puri and A.K. Singh, 2002. Effects of auxins (IBA and NAA) and seasons rooting of juvenile and mature hardwood cuttings of *Robinia pseudoacacia* and *Grewia optiva*. *New Forest*, 2: 143-157.