EEET ECOLOGICAL ENGINEERING & ENVIRONMENTAL TECHNOLOGY

Ecological Engineering & Environmental Technology, 2026, 27(4), 283–296
https://doi.org/10.12912/27197050/219144
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Received: 2026.02.21
Accepted: 2026.03.17
Published: 2026.04.01

An efficient in-vitro micropropagation for *Helianthemum* host plants: A sustainable strategy for desert truffle agroecosystems

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ABSTRACT

Helianthemum guttatum Mill and *Helianthemum ledifolium* are herbaceous *Cistacea* species, known for their drought tolerance and symbiotic association with desert truffles. Even if their propagation mainly occurs through seeds. However, germination is limited by integument-induced physical dormancy. Micropropagation may provide an effective alternative for overcoming this constraint. The present study aimed to develop an *in vitro* regeneration method to facilitate large-scale production of host plants for sustainable truffle cultivation. Seeds collected from native Moroccan populations were subjected to mechanical scarification to enhance germination. Germination and shoot proliferation of axillary and apical buds were evaluated on Murashige and Skoog (MS) basal medium supplemented with different concentrations of 6-benzylaminopurine (BAP: 0, 0.5, 1 and 2 mg.L^{-1}). A maximum germination rate of 100% was observed in scarified seeds cultured on MS or agar media. Shoot proliferation was highest in BAP-free medium, reaching 100% and 95% regeneration from axillary and apical buds, respectively. Root induction was also optimal in the absence of BAP, with 100% for both species. Higher BAP concentration (1–2 mg.L^{-1}) promoted callus formation at the expense of shoot elongation and rooting. Acclimatized plantlets showed survival rates of 90% for *H. ledifolium* and 100% for *H. guttatum*. This optimized protocol provides a reliable system for large-scale propagation of *Helianthemum* species, ensuring a sustainable supply of host plants for desert truffle inoculation in arid and semi- arid Mediterranean regions.

Keywords: BAP, *Helianthemum guttatum* Mill, *Helianthemum ledifolium*, rooting, shoot regeneration.

INTRODUCTION

The genus *Helianthemum* (*Cistaceae*), commonly known as rockroses or sunroses, comprises nearly 110 annual and perennial species widely distributed throughout distributed across the Mediterranean basin (Khabar, 2016; Martín-Hernanz et al., 2021). These species exhibit remarkable adaptability to diverse environmental conditions due to their phenological flexibility, tolerance to various soil substrate, self-compatibility, and ecological plasticity (Rodriguez-Pérez, 2005). *Helianthemum* species are characterized by is their ability to establish symbiotic relationships with mycorrhizal fungi, particularly desert truffles (*Terfezia*, *Tirmania*, *Picoa*). These mycorrhizal interactions enhance nutrient and water uptake in nutrient-poor, drought-prone soils, thereby promoting plant survival in arid ecosystems. Additionally, this symbiotic trait facilitates the production of edible truffle ascocarps, which are of significant economic value in the Mediterranean and Middle East (Khabar, 2014; Zitouni-Haouar et al., 2014; Khabar, 2016; Henkrar et al., 2023).

Among these species, *Helianthemum guttatum* Mill and *Helianthemum ledifolium* (L.) Mill. are two annual plants native to arid and semi-arid zones of Morocco, where they thrive in sandy and rocky soils. They represent important host plants in emerging desert truffle orchards (Henkrar et al., 2023). However, their conventional propagation remains challenging. Seeds-based propagation is often limited by morphological or physical dormancy and reduced viability under nursery conditions, accompanied by high post-germination mortality (Morte et al., 2009). Asexual propagation through cuttings also proven inefficient due to poor rooting and low survival rates as already highlighted by Hamza et al., (2013). To overcome these limitations and meet the increasing demand for standardized host plants in mycorrhizal cultivation systems, *in vitro* micropropagation represents a promising alternative. This approach enables rapid clonal multiplication of selected genotypes while reducing pressure on wild populations (Sarasan et al., 2006; Singh, 2015). The efficiency of micropropagation depends on several factors including genotype, explant source, medium composition, and plant growth regulators. Among these, BAP (6-benzylaminopurine), a synthetic cytokinin, widely used to stimulate shoot induction, although its optimal concentrations varies depending on the species (San José et al. 2012; Ayala et al., 2019; Jayprakash et al. 2021; Meyad et al. 2023). Therefore, the study aimed to develop a reproducible and efficient *in-vitro* micropropagation protocol for *H. guttatum* and *H. ledifolium* by evaluating seed scarification methods and optimizing BAP concentrations for plant regeneration. This work aims to support the production of high-quality host plants for desert truffle cultivation and ecological restoration in arid regions.

MATERIALS AND METHODS

Plant material

Seeds of *H. guttatum Mill* and *H. ledifolium* were collected from two native Moroccan populations: *H. guttatum* from Ain Johra (34°6′0″ N 6°21′0″ W) and *H. led folium* Outat El Haj (33°20′44″ N 3°41′38″ W) between June and August. Mature seeds were stored at room temperature in dry conditions until use.

Seed surface sterilization and scarification

Seeds were first inspected under a stereomicroscope and carefully cleaned to remove plant residues. To overcome physical dormancy, the seed coat was gently scarified using sandpaper. Seeds surface sterilization was carried out under a laminar flow hood. Seeds were immersed in 70% ethanol for 1 minute, followed by 15 minutes in 25% (v/v) commercial bleach solution containing 0.1% Tween-20 under constant agitation. Finally, the seeds were rinsed three times with sterile distilled water to remove any residual sterilizing agents.

Seed germination

Scarified and non-scarified seeds of *H. guttatum* and *H. ledifolium* were cultured on two different media: agar medium (0.6%) and Murashige and Skoog (MS) basal medium supplemented with 15 $g.L^{-1}$ sucrose and solidified with 7 $g.L^{-1}$ bacteriological agar. The pH of both media culture was adjusted to 5.7 ± 0.1 prior to autoclaving at 121 °C for 20 minutes at 1atmosphere of pressure. For each species, twenty seeds were sown per plate, with ten replicate plates prepared for each treatment. Plates were incubated in a controlled growth chamber set at 25 ± 2 °C with a 16-hour photoperiod (light intensity: 40 $\mu mol.m^{-2}.s^{-1}$) and 70% relative humidity. Seed germination was monitored over a five weeks period, and germination percentage was calculated based on the number of visibly germinated seeds (Table 1).

Shoot multiplication and culture conditions

Seedlings at the 1–2 leaf stage were used as explant sources. Axillary buds and apical shoots (1–2 cm in length) were excised and cultured on MS media supplemented with BAP at 0, 0.5, 1.0, or 2.0 $mg.L^{-1}$. Each treatment consisted of 20 explants per replicate, with three independent replicates. Cultures were maintained in 250 mL glass jars containing 50 mL of culture medium and incubated under the same controlled growth chamber conditions described above. Shoot regeneration percentage, number of neo-formed shoots per explant, and callus formation were evaluated after 2 and 5 weeks.

Rooting and callus induction

Rooting performance was evaluated using regenerated shoots cultured on BAP-free MS

Table 1. Effect of different media on the percentage *in vitro* germination of *Helianthemum* seeds – the percentage of germination was recorded after 30 days of seed culture

Media		MS		Agar	
Pre-treatment		Without abrasion	With abrasion	Without abrasion	With abrasion
Germination response (at 4 weeks) (%)	*Helianthemum ledifolium*	45	100	4	1
	Helianthemum guttatum	0	2	4	100

Note: *MS* Murashige and Skoog.

medium and on MS media supplemented with BAP at 0.5 mg.L^{-1}. Root initiation time, rooting percentage, and root length were measured after 2 and 5 weeks of culture. For callus induction studies, thickened or proliferating tissue from explants cultured on 1.0 and 2.0 mg.L^{-1} treatments were transferred to either full-strength or half-strength MS medium (devoid of PGRs) to assess differentiation. Callus-derived regeneration percentage was evaluated after 60 days.

Acclimatization of plantlets

Rooted plantlets were carefully removed from culture vessels, washed to remove agar, and transferred to 150 mL pots containing a sterile peat: perlite mixture (2:1, v/v). Pots were placed in a growth chamber at 25 ± 2 °C under 16 h photoperiod and 80% relative humidity for 4 weeks. Humidity was progressively reduced over time. Plants were irrigated twice a week with half-strength MS nutrient solutionto avoid dehydration of plants and ensure the success of acclimatization. Afterward, acclimatized plants were transferred to a greenhouse with natural lighting and ambient conditions (25 ± 2 °C), where survival rate and morphological traits were monitored.

Experimental design and statistical analysis

All treatments were arranged in a completely randomized design. Each treatment included at least 20 explants per replicate, and each experiment was repeated three times. Data are presented as means ± standard error (SE). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's HSD post hoc test for mean comparison using STATISTICA v10 (StatSoft Inc., USA). Differences were considered statistically significant at $p \leq 0.05$.

RESULTS

In vitro germination of *Helianthemum* seeds

The germination response of *H. ledifolium* and *H. guttatum* seeds was significantly affected by both scarification treatment and culture medium. In *H. guttatum*, mechanical scarification

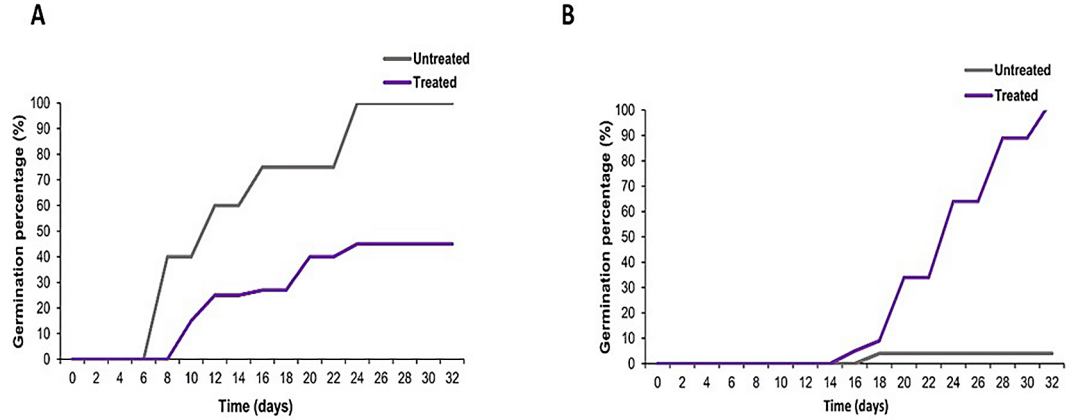

Figure 1. Kinetics of seed ge1n1ination in *Helianthemum ledifplium* and *Helianthemum guttatum* under the effect of abrasion (treated) during 4 weeks. (A) *Helianthemumledifplium,* (B) *Helianthemum guttatum.* The untreated plot consisted of seeds with intact seed coats

followed by culture on agar plates resulted in a 100% germination rate within 30 days. In contrast, non-scarified seeds failed to germinate on MS medium and reached only 4% germination on agar. For, *H. ledifolium,* the highest germination rate (100%) was observed when scarified seeds were cultured on MS medium. Non-scarified seeds of this species exhibited very low germinated rates, ranging from 1 to 4% depending on the medium. Scarified seeds of *H. ledifolium* initiated germination at day 5 and reached maximum germination by day 24, with 90% of seedlings developing leaflets and root primordia. For *H. guttatum*, germination onset was delayed, beginning on day 6 post-culture and reaching nearly 100% by day 30 (Table 2).

Shoot multiplication

Shoot proliferation from axillary buds and apical explants was significantly influenced by both BAP concentration, and explant type, with clear interspecific variation between the two species. The highest shoot regeneration percentages were consistently observed on BAP-free MS medium. For *H. ledifolium*, 100% of axillary buds and 95% of apical segments produced shoots after five weeks of culture (Table 2), similarly, *H. guttatum* showed high regeneration rates of 100% and 95% for axillary and apical explants, respectively (Table 3). The addition of 0.5 mg.L^{-1} BAP resulted in a marked decrease in shoot regeneration efficiency. In *H. ledifolium*, regeneration rates declined to 75% for axillary buds and 85% for apical segments. The reduction was more pronounced in *H. guttatum,*, with regeneration rates decreasing to 70% for axillary buds and 60% for apical explants. Moreover, the average number of newly formed shoots per explant was significantly lower in BAP-supplemented media compared to 1.6 ± 0.84 shoots at 0.5 mg. L^{-1}BAP- free medium. Similar trends were observed for *H. guttatum* (Table 4). Statistical analysis confirmed that these differences were significant ($p \leq 0.05$).

Table 2. Effect of BAP on shoot regeneration, rooting percentages and callus induction of *H. Ledifolium* – data was collected after 2 and 5 weeks of culture, experiments with 20 replicates

Parameter	Plant growth regulators (mg/l)		Shoot regeneration (%)		Rooting percentage (%)		Callus induction (%)	
			At 2 wk	At 5 wk	At 2 wk	At 5 wk	At 2 wk	At 5 wk
Axillary buds	Control	0.0	30%	100%	35%	100%	0%	0%
	BAP (mg L^{-1})	0.5	20%	75%	20%	55%	0%	0%
		1.0	0%	0%	0%	0%	15%	80%
		2.0	0%	0%	0%	0%	25%	100%
Apical	Control	0.0	30%	95%	50%	95%	0%	0%
	BAP (mg L^{-1})	0.5	25%	85%	25%	50%	0%	0%
		1.0	0%	0%	0%	0%	10%	60%
		2.0	0%	0%	0%	0%	30%	100%

Note: *BAP* 6 Benzylaminopurine,*mg/l* milligram per liter, *wk* weeks.

Table 3. Effect of BAP on shoot regeneration, rooting percentages, and on callus induction of *H. guttatum* – data was collected after 2 and 5 weeks (wk) of culture, experiments with 20 replicates

Parameter	Plant growth regulators (mg/l)		Shoot regeneration (%)		Rooting percentage (%)		Callus induction (%)	
			At 2 wk	At 5 wk	At 2 wk	At 5 wk	At 2 wk	At 5 wk
Axillary buds	Control	0.0	25%	100%	35%	100%	0%	0%
	BAP (mg L^{-1})	0.5	20%	75%	0%	55%	0%	0%
		1.0	0%	0%	0%	0%	30%	85%
		2.0	0%	0%	0%	0%	40%	100%
Apical	Control	0.0	20%	95%	10%	95%	0%	0%
	BAP (mg L^{-1})	0.5	0%	70%	25%	40%	0%	0%
		1.0	0%	0%	0%	0%	25%	90%
		2.0	0%	0%	0%	0%	45%	100%

Note: *BAP* 6 Benzylaminopurine,*mg/l* milligram per liter.

Table 4. The effect of MS medium with different concentrations of BAP on neoformed shoots and cultivated plant of each explants of *H.ledifolium* and *H. guttatum*, from axillary buds and apical buds after 5 weeks of culture

Parameter	Plant growth regulators (mg/l)		*Helianthemum ledifolium*		*Helianthemum guttatum*	
			Neoformed shoots per explants ± SE	Cultivated explants per explants ± SE	Neoformed shoots per explants ± SE	Cultivated explants per explants ± SE
Axillary buds	Control	0.0	3 ± 0**	3 ± 0**	3 ± 0**	2.9 ± 0.31**
	BAP (mg L^{-1})	0.5	1.6 ± 0.84*	1± 0.66*	1.9± 0.56*	1.5± 0.52*
		1.0	0	0	0	0
		2.0	0	0	0	0
Apical	Control	0.0	2.7 ± 0.48**	2.6± 0.51**	2.8± 0.42**	2.6± 0.51**
	BAP (mg L^{-1})	0.5	1.4 ± 0.84*	1± 0.81*	2.1± 0.56*	1.1± 0.73*
		1.0	0	0	0	0
		2.0	0	0	0	.0

Note: Each statistic signifies the average ± standard error of three experiments with 10 replicates. Means ± SE within, followed by the same number *, are not significantly different under the HSD Tukey test at $p \leq 0.05$. *BAP* 6 Benzylaminopurine,*mg/l* milligram per liter.

Exposure to higher BAP concentrations (1.0 and 2.0 mg.L^{-1}) completely inhibited shoot regeneration in both species. Instead, thick green calluses developed at the base of the explants, with callus induction reaching 100% in *H. guttatum* and up to 90% in *H. ledifolium* (Tables 2 and 3). These results indicate a dose-dependent effect of BAP, where elevated cytokinin levels suppress organogenesis and promote callogenesis. Distinct morphological differences were observed among treatments. Shoots regenerated on BAP-free were elongated, vigorous, and morphologically uniform. In contrast, explants cultured in the presence of 0.5 mg·L^{-1} BAP were shorter shoots that frequently exhibited basal swelling or tissue thickening. This inhibitory effect on shoot elongation become more pronounced as BAP concentrations increased. These morphological variations are illustrated in Figures 2 and 3, which show the progressive inhibition of shoot development and the induction of callus formation as BAP concentration increases from 0 to 2 mg·L^{-1}. Explant type also played a crucial role in shoot multiplication. Axillary buds consistently outperformed apical segments in terms of regeneration rate, shoot number, and shoot length across all treatments. Collectively, these results demonstrate that a BAP-free MS medium represents the optimal condition for shoot induction and proliferation in both *Helianthemum* species, there by providing a strong foundation for efficient *in vitro* plant propagation.

Callus induction and regeneration

Callus formation was strongly induced in both *H. guttatum* and *H. ledifolium* when explants were cultured on MS medium supplemented with 1.0 or 2.0 mg·L^{-1} BAP. In *H. guttatum*, 85% and 100% of axillary bud explants formed calluses at 1.0 and 2.0 mg·L^{-1} BAP, respectively, while apical segments exhibited callus induction rates of 90% and 100% under the same conditions (Table 3). Similar responses were observed in *H. ledifolium*, with callus formation ranging from 80 to 100% in both explant types (Table 2). In contrast, no callus development occurred in BAP-free or 0.5 mg·L^{-1} BAP conditions.

Macroscopically, the calluses were compact and green, with moderate initial swelling observed at 1.0 mg·L^{-1} BAP. At 2.0 mg·L^{-1}, calluses became denser and more voluminous but no further organogenic development was observed during the initial culture phase (Figure 2c,d; 3c,d). To evaluate their regenerative potential, calli obtained from explants cultured on 1.0 mg·L^{-1} BAP were transferred to full-strength (MS) and half-strength MS (½ MS) media without growth regulators. Regeneration rates varied by species and medium. In *H. ledifolium*, shoot regeneration from callus reached 43% on MS and 75% on ½ MS medium, while acclimatization success rates were 63% and 74%, respectively (Table 5). For *H. guttatum*, regeneration was higher, reaching 55% on MS and 66% on ½ MS, with subsequent acclimatization rates of 75% and 80%. These results demonstrate that although elevated BAP concentrations effectively

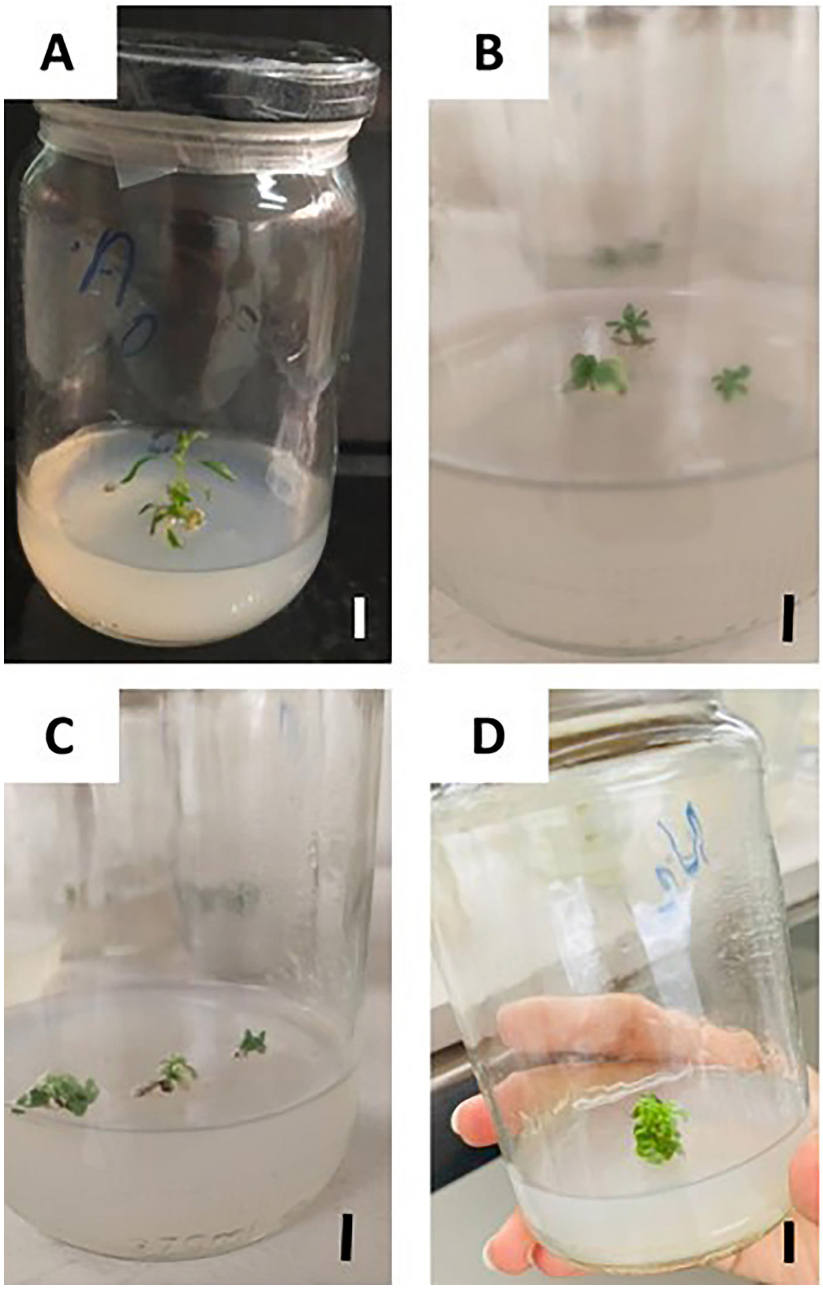

Figure 2. Micropropagation of *Helianthemum ledifolium* from the axillary bud and the apices derived from the plant results from in vitro germination, after 5 weeks of culture on:
(a) Murashige and Skoog (MS) medium without growth regulator.
(b) Murashige and Skoog (MS) medium supplemented with 0.5 mg.L^{-1} BAP.
(c) Murashige and Skoog (MS) medium supplemented with 1 mg.L^{-1} BAP.
(d) Murashige and Skoog (MS) medium supplemented with 2 mg.L^{-1} BAP. Black bar = 1 cm

Table 5. Indirect shoot regeneration response from calls obtained from different explants in *H. ledifolium* and *H. guttatum* on media supplemented with 1 mg. L^{-1} of BAP

Parameter	*Helianthemum ledifolium*		*Helianthemum guttatum*	
	Percentage of shoots regenerated per calls	Percentage of acclimated plants	% of shoots regenerated per calls	Percentage of acclimated plants
MS	43	63	55	75
½ MS	75	74	66	80

Note: *MS* Murashige and Skoog.

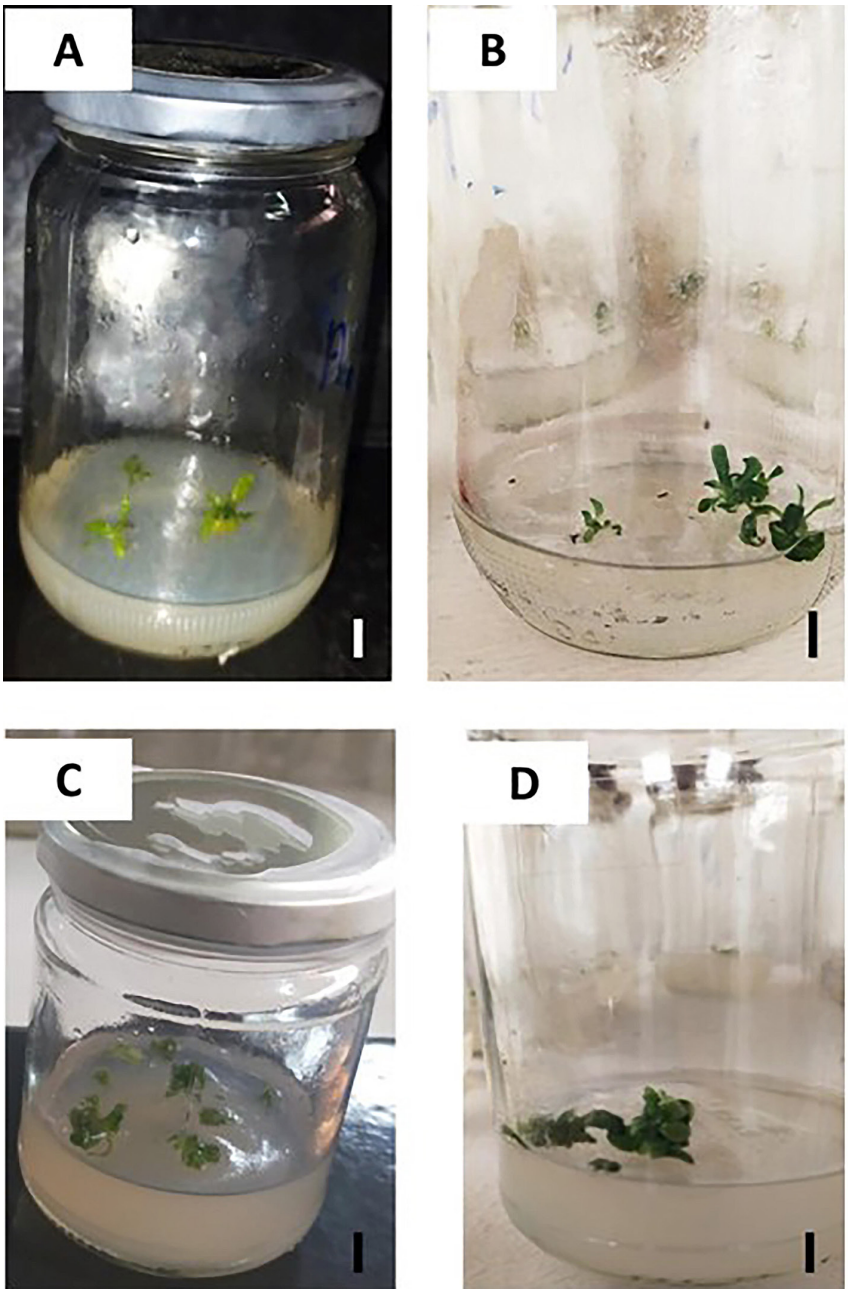

Figure 3. Micropropagation of *Helianthemum guttatum* derived the plants' results from in vitro germination culture, after 5 weeks of culture on (a) Murashige and Skoog (MS) medium without growth regulator. (b) Murashige and Skoog (MS) medium supplemented with 0.5 mg.L^{-1} BAP. (c) Murashige and Skoog (MS) medium supplemented with 1 mg.L^{-1} BAP. (d) Murashige and Skoog (MS) medium supplemented with 2 mg.L^{-1} BAP. Black bar = 1 cm

induce callogenesis, optimal organogenic regeneration occurs during secondary culture under hormone-free conditions, particularly when nutrient strength is reduced.

Rooting and plantlet development

Root induction occurred spontaneously in both *Helianthemum* species when regenerated shoots were cultured on hormone-free MS medium. Under this condition, *H. guttatum* and *H. ledifolium* achieved maximum rooting percentages of 100% and 95%, respectively, regardless of explant origin (Tables 2 and 3). Root initiation began as early as 15 days post-culture in *H. ledifolium*, whereas *H. guttatum* exhibited a slower rooting response, with root emergence occurring between days 18 and 24. In contrast,

shoots cultured on medium supplemented with 0.5 mg·L^{-1} BAP exhibited significantly lower rooting rates, anging from 40% to 55% depending on species and explant type. Additionally, BAP-treated explants often formed small basal calluses, and the roots that developed were fewer, shorter, and less vigorous compared to those in hormone-free conditions. Quantitative analysis further revealed, that both length of roots and aerial parts were significantly greater in axillary-derived shoots than from apical explants across all treatments. On BAP-free MS medium, axillary-derived shoots of *H. guttatum* and *H. ledifolium* reach droot lengths of 26.75 ± 1.36 cm and 21.41 ± 2.36 cm, respectively (Figure 5A), while roots apical-derived shoots were slightly shorter (Figure 5B). A similar trend was observed for shoot length, where axillary explants consistently produced longer aerial structures (Figure 4). Statistical analysis confirmed that these differences were significant ($p \leq 0.05$), reinforcing the role of explant source and hormone-free media in successful plantlet development.

Acclimatization and plant survival

Following rooting, regenerated plantlets were successfully acclimatized to ex vitro conditions. Rooted plants were transferred to a peat: perlite (2:1) mixture and maintained under high humidity before gradual transition to greenhouse conditions. Both species showed high survival rates after acclimatization: 100% in *H. guttatum* and 90% in *H. ledifolium* (Figure

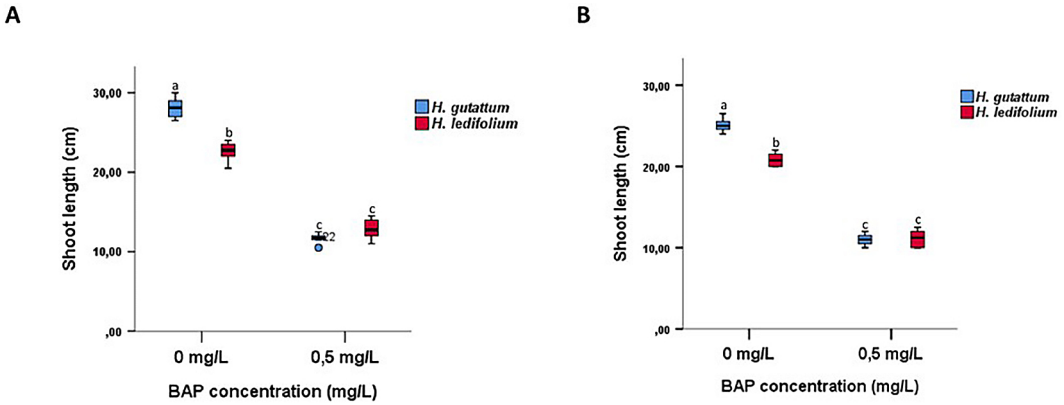

Figure 4. Box plot showing shoot length in response to different BAP concentration (mg.L) after 8 weeks of plantlet acclimatization of *Helianthemum ledifolium* and *Helianthemum guttatum*. (A) Axillary buds. (B) Apical part. According to Tukey's HSD test, a, b, c indicate significant differences for each BAP concentration (mg.L^{-1})

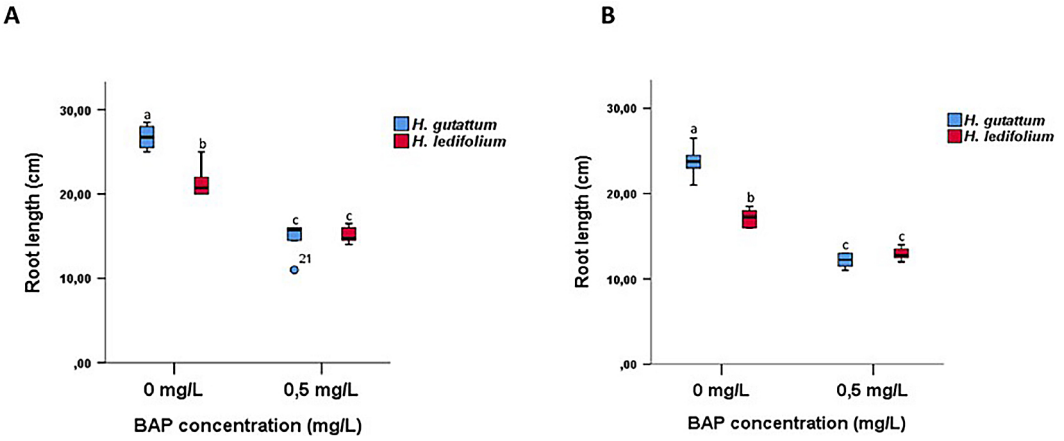

Figure 5. Box plot showing root length in response to different BAP concentrations (mg.L^{-1}) after 8 weeks of plantlet acclimatization of *Helianthemum ledifolium* and *Helianthemum guttatum*. (A) Axillary buds. (B) Apical part. According to Tukey's HSD test, a, b, c indicate significant differences for each BAP concentration (mg.L^{-1})

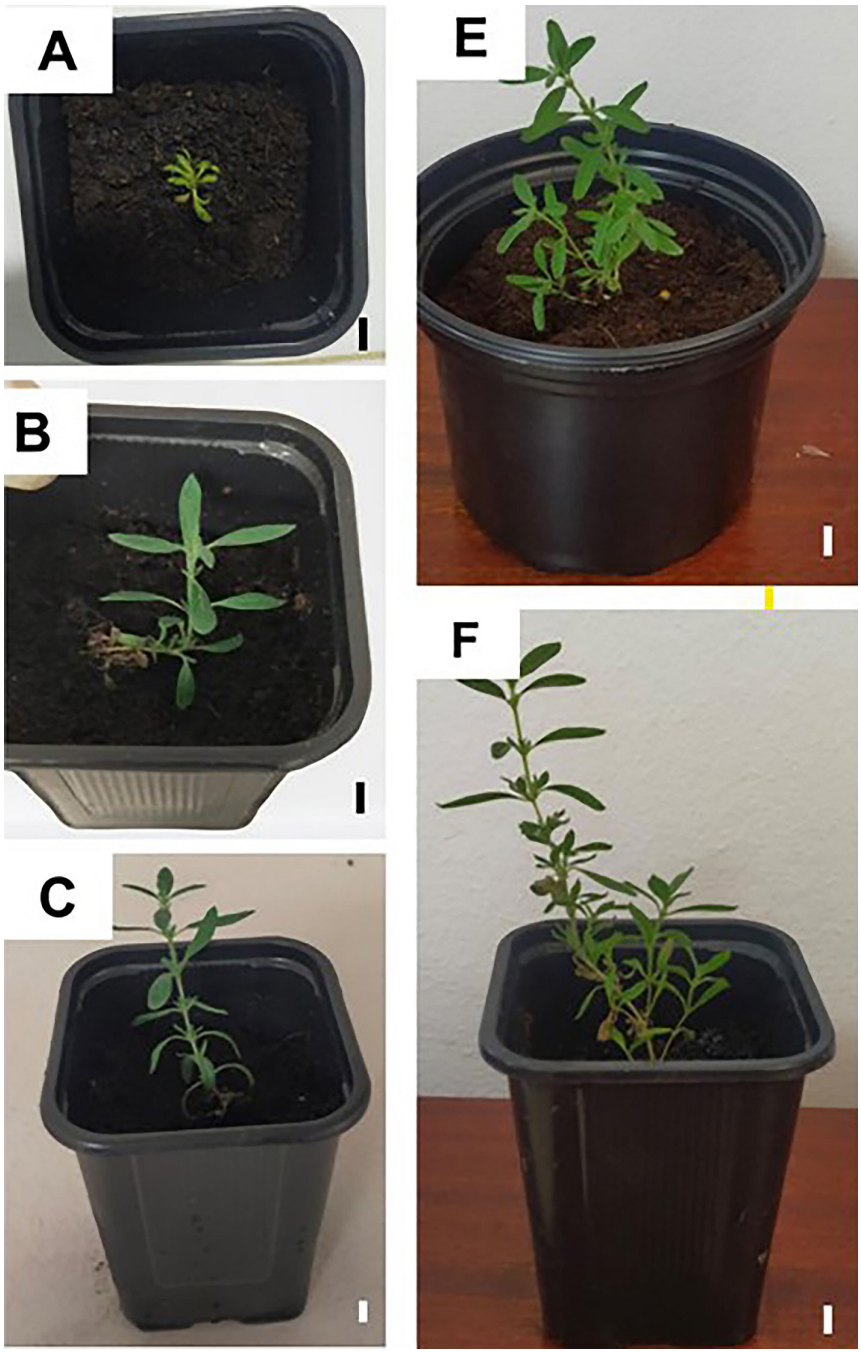

Figure 6. General aspect of *Helianthemum ledifolium* plants during 8 weeks of acclimatization (A). The first day of transferring the explants into a plastic pot. (B) Development of acclimated plants after two weeks of transfers to the pot. (C) Plants after four weeks of acclimatization. (D) Development of the plant after six weeks of acclimation. (E) Plantlet after eight weeks of acclimation. White bar = 1 cm

6 and 7). No morphological abnormalities or phenotypic deviations were observed among regenerated plants, suggestion the genetic stability of the micro propagated material.

Morphometric analysis conducted during acclimatization revealed that plantlets derived from axillary buds consistently developed longer shoots and roots than those from apical segments, particularly when initially cultured on hormone-free media. Overall, these results underscore the robustness of the established micropropagation protocol and confirm its suitability for large-scale plant production and subsequent field establishment.

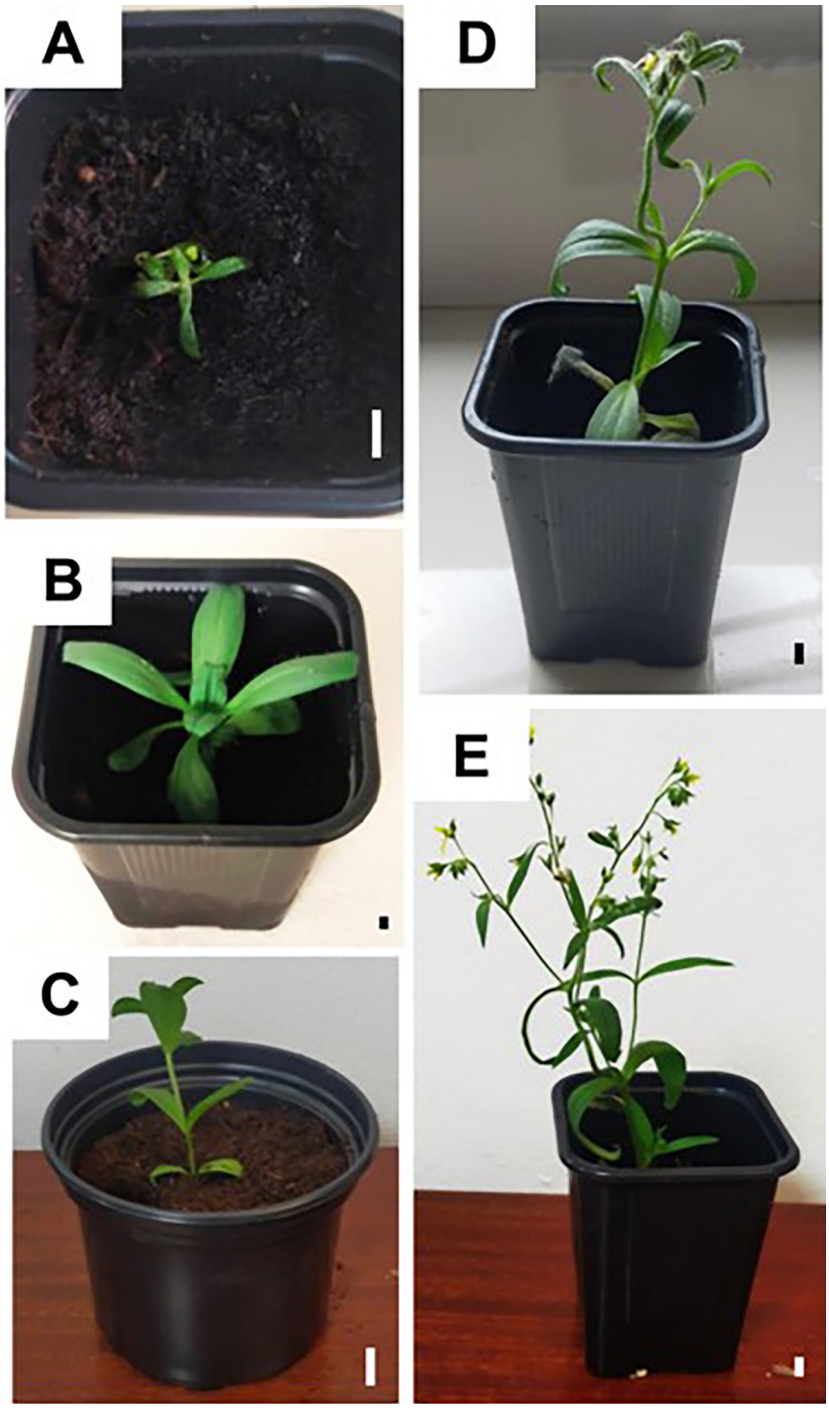

Figure 7. General aspect of *Helianthemum guttatum* plants during 8 weeks of acclimatization (A) The first day of transferring the explants into a plastic pot. (B) Development of acclimated plants after two weeks of transfers to the pot. (C) Plants after four weeks of acclimatization. (D) Development of the plant after six weeks of acclimation. (E) Plantlet after eight weeks of acclimation. White bar = 1 cm

DISCUSSION

The establishment of mycorrhization program is fundamental for the sustainable production of desert truffles and the ecological restoration of degraded Mediterranean areas. However, a major limitation in implementing such programs lies in the availability of large quantities of uniform, disease-free and standardized plant material. Plant tissue culture techniques provide an effective solution, enabling rapid clonal propagation of selected

individuals while ensuring production of true-to-type plants (Louro et al., 2020).

The present study successfully developed a reproducible and efficient micropropagation protocol for *H. guttatum* and *H. ledifolium*, two ecologically significant host species for desert truffles cultivation. This protocol overcomes key limitations associated with conventional propagation methods, such as low germination rates, high integumentary dormancy, and poor rooting ability of cuttings, challenges previously reported for *Helianthemum* and other *Cistaceae* genera (López et al., 2004; Morte et al., 2009; Hamza et al., 2013). Mechanical scarification proved essential for breaking seed dormancy, particularly in *H. guttatum*, where non-scarified seeds failed to germinate. These findings supports previous reports indicating that impermeable seed coats act as a primary dormancy mechanism in *Helianthemum* species (Rodríguez-Pérez, 2005; Pérez-García et González-Benito, 2006). The species-specific response to culture media, with *H. ledifolium* growing better on MS medium and *H. guttatum* on agar plates, suggests differences in nutritional requirements or hormonal balance during early development. Such variability likely reflects ecological adaptations to distinct microhabitats and emphasizes the necessity of species-specific germination strategies.

Shoot multiplication was most effective on hormone-free MS medium, indication minimal dependence on exogenous cytokinins. This suggests that axillary buds, possess sufficient endogenous hormonal levels to sustain organogenesis. The suppressive impact of BAP, even at low concentrations, and inhibitory effect at higher concentrations, are consistent with previous reports in *Helianthemum almeriense, Helianthemum violaceum* and *H. marminorense* (Morte and Honrubia, 1997; Morte et al., 2009; Serrano-Matinez et al., 2012). These studies show the importance of hormone balance, as excessive cytokinin exposure can disrupt the equilibrium between cell division and differentiation, tending to induce callogenesis rather than shoot induction (Ikeuchi et al., 2013; Jayaprakash et al., 2021).

Callogenesis was pronounced at higher BAP concentrations, although it regenerative capacity varied. Notably, transferring calli to hormone-free or half-strength MS medium significantly enhanced shoot regeneration. This suggests that the reduced salt strength and the absence of growth regulators may favor redifferentiation by alleviation hormonal stress and promoting organogenesis pathways. These results are in agreement with earlier observations published in woody perennials and Mediterranean endemic species, where half-strength and hormone-free media improved shoot growth from callus (Behera and Sahoo, 2009; Dönmez et al., 2022). Rooting was also improved in hormone-free media, thus confirming the inhibitory influence of cytokinins on root induction. Both *H. guttatum* and *H. ledifolium* showed spontaneous rooting in the absence of auxins, a trait likely associated with the adaptation to arid environmental, where rapid root development enhances survival. The earlier onset and greater root length in *H. ledifolium* indicate species-dependent physiological or anatomical variations in root formation, likely with their different ecological microhabitats. Importantly, natural rooting reduces production cost and simplifies large-scale propagation for conservation and truffle Orchard applications. The superior performance of axillary buds compared to apical segments in terms of shoots number, elongation, and rooting aligns with previous studies highlighting greater meristematic activity and endogenous hormone content of axillary tissues (Molnar et al., 2011, San José et al., 2021). This finding is particularly relevant regarding the standardization of protocols, as axillary buds are easier to isolate, more uniformed, and develop stronger plantlets. High acclimatization rates (90–100%) and the lack of phenotypic abnormalities confirm the stability and physiological integrity of the propagation system. Successful transition from *in vitro* to *ex vitro* conditions validates their predicted compatibility with mycorrhizal symbiosis. Given that successful desert truffle cultivation depends on establishing compatible and vigorous host plants, this method offers evaluable foundation for the development of desert truffle orchards in Moroccan (Morte et al., 2021; Henkrar et al., 2023).

Overall, this study contributes to the biotechnological advancement of *Helianthemum* species and supports the conservation of Mediterranean dryland ecosystems. Clonal propagation of mycorrhizal-compatible hosts may reduce pressure on wild populations, facilitate habitat restoration and promote sustainable agroecological innovation through desert truffle farming.

Further studies should explore the integration of mycorrhizal inoculation under controlled conditions to evaluate symbiotic and functional impacts. Additionally, assessment of genetic fidelity using molecular tools such as SSR or RAPD markers well be important to guarantee clonal uniformity for large-scale agronomic applications.

CONCLUSIONS

This research reports a reliable and reproducible *in vitro* micropropagation protocol for *H. guttatum* and *H. ledifolium*, two ecologically and economically relevant host plants for desert truffle cultivation in Mediterranean arid regions. Mechanical scarification effectively overcomes seed dormancy, particularly in *H. guttatum*. Optimal shoot regeneration and rooting were achieved on a cytokinin-free MS medium, with axillary buds showing higher morphogenetic potential than apical segments. High BAP concentrations induced callus formation which prevented shoot proliferation, emphasizing the importance of hormonal balance in improving regeneration rates. Callus-derived shoots could be successfully regenerated and acclimatized on hormone-free, half-strength MS medium, indicating the feasibility of indirect organogenesis. Both species rooted naturally without adding auxin, and acclimatization success exceeded 90%, confirming the physiological integrity of the regenerated plantlets. The protocol provides a scalable, cost-effective strategy for the mass production of clone-plants, representing a significant step toward sustainable desert truffle cultivation. Future research should focus on the controlled incorporation of mycorrhizal fungi and molecular validation of clonal fidelity, to further validate the use of this protocol in ecological and agronomic applications.

Acknowledgments

All experiments were done at Mohammed V University, Rabat, Morocco, Faculty of Sciences, Department of Biology, Laboratory of Botany and Valorisation of Plant and Fungal Resources.

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