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Micropropagation of Helichrysum Arenarium (L.) Moench Subsp. Erzincanicum: an Endemic Plant Species

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ABSTRACT

The objective of this research was to establish a micropropagation protocol through the use of explants grown from seeds of Helichrysum arenarium (L.) Moench subsp. erzincanicum and plant tissue culture methods. Initially, indirect shoot organogenesis was performed with H. arenarium subsp. erzincanicum in vitro plantlets on Murashige and Skoog (MS) and Gamborg B5 media (abbrev. as MSB) supplemented with 6-benzylaminopurine (BAP), kinetin (Kin) and indole-3-acetic acid (IAA). A maximum number of shoots was induced in media supplemented with 1 mg L-1 BAP. Subsequently, the shoots were inoculated in MSB combinations with 0.5-1.5 mg L-1 indole-3-acetic acid and indole-3-butyric acid (IBA), the highest root proliferation was recorded with 1.0 mg L-1 IBA (77.8%). Additionally, callus formation was achieved with various doses of TDZ (thidiazuron) (0.5, 1.0, 2.0 mg L-1) and α-naphthaleneacetic acid (NAA) (1.0 mg L-1) and the highest callus formation percentage was observed MSB media combinations with 2.0 mg L-1 TDZ and 1.0 mg L-1 NAA. This study presents the successful development of a micropropagation protocol for Helichrysum arenarium subsp. erzincanicum and supports its in vitro propagation via explants grown from seed. Hence, We aimed to develop an efficient micropropagation protocol was established for this subspecies using explants derived from seeds endemic to and naturally occurring in Türkiye. This micropropagation protocol starting from seeds provides an alternative to clonal propagation and contributes to the conservation of Helichrysum arenarium (L.) Moench subsp. erzincanicum a rare and endemic plant species. Furthermore, this research contributes to the formulation of in vitro methods for propagating other Helichrysum and rare plant species.

***Keywords*:** Endemic species, Helichrysum arenarium subsp. erzincanicum, Gibberellic acid, Kinetin, Micropropagation, Seed.

INTRODUCTION

Helichrysum arenarium (L.) Moench subsp. erzincanicum P.H. Davis & Kupicha is endemic to Türkiye, specifically distributed across the provinces of Kastamonu, Ağrı, Erzincan, and Kayseri. It is a perennial, herbaceous species endemic to Türkiye, growing in its natural vegetation [1, 2]. In Türkiye, 30 Helichrysum species have been identified, 16 of which are endemic [3]. Helichrysum is also commonly known as golden grass, an everlasting or immortal flower [4]. Helichrysum has traditionally been used as a diuretic, cholagogue, hepatoprotective effect, bile-production, anti-clotting and capillary-stabilizing agent with effects in the gastrointestinal tract, liver and gallbladder [5].

Many Helichrysum species have been used not only in folk medicine but also for ornamental, cosmetic, and pharmaceutical purposes [6, 7]. With its bright yellow color, it is suitable for fresh and dry bouquet arrangements. Essential oil and aromatic chemical glycosides are utilized for the cosmetics, food pharmaceutical and industries as insect repellents [8]. The essential oils and phytochemicals of Helichrysum, such as monoterpenoids, sesquiterpenes, triterpenoids, flavonoids, and pyrones, have important pharmaceutical properties [9, 10]. Because of its antiproliferative, anti-inflammatory, antiviral, antifungal, and antimicrobial effects, Helichrysum species need to be protected and produced, especially by in vitro methods. Micropropagation is an efficient plant tissue culture method that has been applied to a very wide range of species. Micropropagation techniques are important in terms of preserving the generation of endemic plants and ensuring their production of bioactive components in vitro.

To date, different Helichrysum species have typically been propagated from axillary buds, leaves, or apical meristems. For example, the apical meristems of H. arenarium were used as explants and determined that the best phytohormone composition for shoot initiation was 5.0 mg L^{-1} N6-furfuryladenine (Kin) and 0.5 mg L^{-1} indole-3-acetic acid (IAA) or 5.0 mg L^{-1} Kin alone [11]. Another research, shoots and stem nodes were utilized as explants for micropropagation of H.

arenarium and found that more calli formed in [12] medium containing 1.0 mg L^{-1} 2,4-D [13]. Besides, the apical buds and axillary shoots of Helichrysum italicum subsp. microphyllum were applied as explant source [14]. Moreover, the young leaves and axillary buds were initiated of H. kraussii in vitro culture and calli formed in MS nutrient media supplemented with 1 mg L^{-1} α-naphthaleneacetic acid (NAA) and 0.05 mg L^{-1} Kin [15]. Furthermore, it has been reported the use of H. italicum axillary buds as explants for shoot regeneration. It has been optimized an in vitro propagation protocol from Helichrysum umbraculigerum leaves [16]; and used young H. italicum shoots for in vitro production. However, to our knowledge, there are no prior reports on the micropropagation of endemic H. arenarium subsp. erzincanicum. Hence, in this project we targeted to establish with optimization an effective micropropagation protocol for this subspecies using explants derived from seeds endemic to and naturally occurring in Türkiye.

Materials and methods

Material

H. arenarium (L.) Moench subsp. erzincanicum Davis & Kupicha Figure 1 (abbreviated as HAE) seeds were collected from the variety garden of the Muş Alparslan University Faculty of Applied Sciences in October 2021. Then it was identified by Dr. Ümit Bingöl (M.Ü. Bingöl 6829) from the Faculty of Science, Ankara University.

Figure 1. Helichrysum arenarium (L.) Moench subsp. erzincanicum Davis & Kupicha aerial parts from the variety garden of the Muş Alparslan University Faculty of Applied Sciences.

Methods

Seed sterilization and germination

Seeds were disinfected with 7.5 % sodium hypochlorite (NaOCl) and placed in an oven at 25°C for 20 minutes. Afterward, it was applied 70 % ethanol 30 seconds and then seeds were incubated at 42°C for 24 hours in the oven. Then, the sterile seeds were washed for five times with double distilled water [17]. After sterilization, the medium was additionally supplemented with 1.0 mg L^{-1} gibberellic acid (GA_3), 1.0 mg L^{-1} 6-benzylaminopurine (BAP), and 1.0 mg L^{-1} amphotericin B. The samples were maintained at room temperature (24 ± 2°C) under white fluorescent light (3000 lux) with a photoperiod of 16 hours of light and 8 hours of darkness. The germination study included three replicates, each containing 20 seeds.

Shoot regeneration media

After 21 days of germination the in vitro-obtained plantlets were transferred to MSB media and used as explants. The following nutrient media mixtures were prepared for shoot regeneration: M1 (1.0 mg L^{-1} BAP); M2 (2.0 mg L^{-1} BAP); M3 (3.0 mg L^{-1} BAP); M4 (1.0 mg L^{-1} BAP and 0.1 mg L^{-1} NAA); M5 (2.0 mg L^{-1} BAP and 0.1 mg L^{-1} NAA); M6 (3.0 mg L^{-1} BAP and 0.1 mg L^{-1} NAA); M7 (1.0 mg L^{-1} Kin and 0.5 mg L^{-1} NAA); M8 (2.0 mg L^{-1} Kin and 0.5 mg L^{-1} NAA); M9 (3.0 mg L^{-1} Kin and 0.5 mg L^{-1} NAA); M10 (4.0 mg L^{-1} Kin and 0.5 mg L^{-1} NAA); and M11 (5.0 mg L^{-1} Kin and 0.5 mg L^{-1} NAA).

Callus growth medium

Calli that formed in media supplemented with 1.0 mg L^{-1} BAP were used as explants for callus culture in MSB media in the presence of various PGRs. The following media mixtures were prepared for callus culture: 2,4-D (0.5 mg L^{-1}, 1.0 mg L^{-1}, 1.5 mg L^{-1}); TDZ (1.0 mg L^{-1}) and picloram (0.5 mg L^{-1}, 1.0 mg L^{-1}, 2.0 mg L^{-1}); NAA (1.0 mg L^{-1}) and TDZ (0.5 mg L^{-1}, 1.0 mg L^{-1}, 2.0 mg L^{-1}).

Rooting of shoots

Eight-week-old shoots were moved to (MS) medium enhanced with 0.1, 0.5 or 1.0 mg L^{-1} IAA or IBA for formation of root

Statistical analysis

Data were subjected to statistical analysis using IBM SPSS for Windows (SPSS Inc., Chicago, IL, USA). Differences in means ± standard errors (SEs) were evaluated for significance using Duncan's one-way analysis of variance (ANOVA) at the $P < 0.05$ level.

RESULTS

Frequency of seed germination

The effects of the PGRs; BAP and GA_3 on the germination of HAE seeds on MSB medium are shown in Table 1. The maximum seed germination rate (27.22%) was achieved in Mursahige and Skoog basal medium containing 1.0 mg L^{-1} GA_3 and 1.0 mg L^{-1} BAP.

Table (1): Effects of the plant growth regulators BAP and GA_3 on the seed germination frequency of HAE in MSB media

Concentration of PGRs		Germination frequency (%)
Control		6.6 ± 1.6^{d}
GA_3 (mg L^{-1})	BAP (mg L^{-1})	
1.0	0.0	21.31 ± 2.1^{b}
1.0	1.0	27.22 ± 2.6^{a}
1.0	2.0	14.76 ± 1.2^{c}

Within each column means labelled with distinct letters represent significant difference between means at the 0.05 level, and (±) denotes the standard error.

Shoot proliferation

The sterile seedlings were transferred to nutrient media containing three different concentrations of growth regulator (0.5, 1.0, and 2.0 mg L^{-1} BAP) at the end of the 21st day Table 2. The optimum rate of shoot organogenesis was recorded on MSB medium enriched with 1.0 mgL^{-1} BAP (61.2%, $P<0.05$).

Table (2): Effects of different concentrations of BAP in MSB media on HAE shoot formation eight weeks after explant initiation of culture

Phytohormones (mg L^{-1})	Rate of shoot induction from the explants (%)
BAP	
0.5	19.8 ± 1.6^{c}
1.0	61.2 ± 4.2^{a}
2.0	$38.0\,3 \pm 2.8^{b}$

Within each column means labelled with distinct letters represent significant difference between means at the 0.05 level, and (±) denotes the standard error.

The adventitious shoots generated from the tissue segments on medium containing 1.0 mg L^{-1} BAP were subcultured to MSB medium supplemented with various treatments of Kin and IAA. By the end of the 30th day the plantlets had grown in height Figure 2.

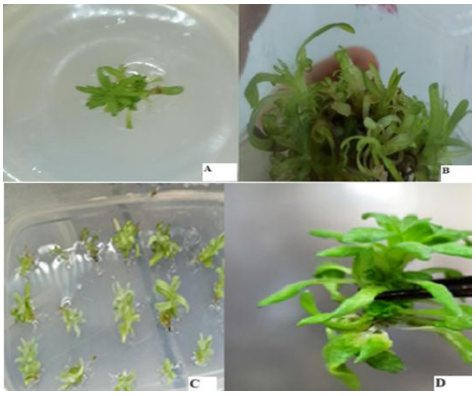

Figure 2. Shoot regeneration (A) on the first day after placing in MSB medium containing 1 mg L^{-1} BAP and (B) transfer of the micro-plants after 8 weeks. (C) Images of plantlets transferred from media enhanced with 1.0 mg L^{-1} BAP to media supplemented with 5.0 mg L^{-1} Kin with 0.5 mg L^{-1} NAA (C) on the first day and (D) after 30 days.

Among the six different combinations of Kin and IAA tested for shoot regeneration, 5.0 mg L^{-1} Kin and 0.5 mg L^{-1} IAA resulted in the maximum ratio of explants with shoot formation, reaching 60% Table 3.

Table (3): Response of shoot formation of HAE on MSB growth medium enriched with different doses of Kin and IAA

Phytohormones combination (mg L^{-1})		Explants with shoot formation (%)
Kin	IAA	
0.0	0.0	26.6 ± 1.3^{c}
1.0	0.5	-
2.0	0.5	-
3.0	0.5	53.3 ± 2.7^{a}
4.0	0.5	46.7 ± 2.3^{b}
5.0	0.5	60 ± 3.1^{a}

Callus formation and structure

The structures and colours of the calli obtained are given in Table 4, and the treatments yielded significantly different frequency of calli formation ($p<0.05$). The maximum ratio of callus induction (80.2%) was derived with MSB medium enriched with 2.0 mgL^{-1} TDZ and 1.0 mgL^{-1} NAA Figure 3.

Table (4): Effects of callus growth of HAE on MSB growth medium enriched with different doses of 2,4-D, TDZ, NAA, and picloram

Phytohormones		Callus structure and colour	Frequency of callus formation (%)
2,4-D (mg L^{-1})			
0.5		Brown, compact	27.1 ± 2.4^{b}
1.0		Brown, friable	38.7 ± 3.1^{a}
2.0		Brown, compact	17.8 ± 1.6^{c}
TDZ (mg L^{-1})	Picloram (mg L^{-1})		
1.0	0.5	Yellowish, compact	26.7 ± 2.8^{a}
1.0	1.0	Brown, friable	33.3 ± 3.1^{a}
1.0	2.0	Brown, compact	20.0 ± 2.4^{b}
TDZ (mg L^{-1})	NAA (mg L^{-1})		
0.5	1.0	Brown, compact	26.7 ± 4.8^{c}
1.0	1.0	Yellowish, friable	60.1 ± 10.2^{b}
2.0	1.0	Greenish, friable	80.2 ± 16.8^{a}

Within each column means labelled with distinct letters represent significant difference between means at the 0.05 level, and (±) denotes the standard error.

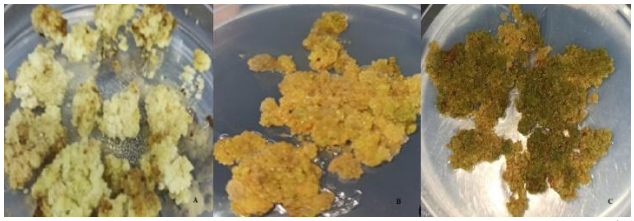

Figure 3. Callus formation after 60 days in MSB media supplemented with (A) 1 mg L^{-1} TDZ and 0.5 mg L^{-1} Pic; (B) 1 mg L^{-1} TDZ and 1.0 mg L^{-1} NAA; and (C) 2 mg L^{-1} TDZ and 1.0 mg L^{-1} NAA.

Rooting

Elongated plantlets were subcultured onto MSB medium containing 0.5, 1.0, or 1.5 mg L^{-1} IBA or IAA for roots developed within four periods of culture. The highest ratio (77.8%) of rooted shoots were found in the 1.0 mg L^{-1} IBA applications group in which new adventitious plantlets formed Figure 4 and Figure 5.

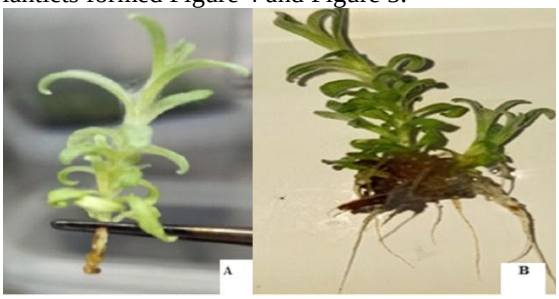

Figure 4. Representative images of plantlets transferred to medium enhanced with 1.0 mg L^{-1} IBA for rooting (A) on the first day and (B) after 30 days.

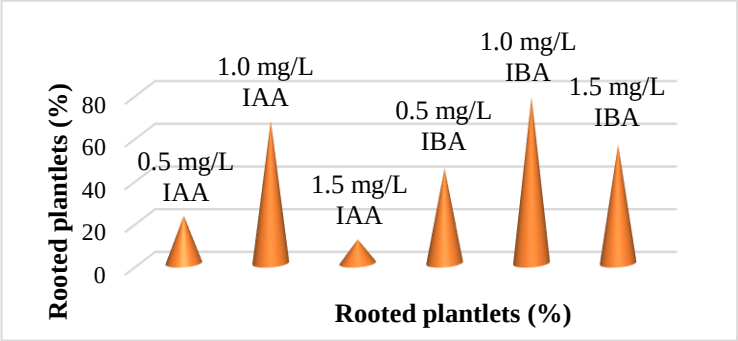

Figure 5. Root formation frequencies (%) after 30 days of HAE seedlings

Discussion

The sterilization of the HAE seeds was challenging due to their small size and hairy structure. **[16]** reported the sterilization method that was successfully applied in this study. While low amounts of sodium hypochlorite (7.5%) did not damage the seeds' structure, increasing the temperature to 42°C removed the bacteria, fungi, and spores that may have been present on the seeds' hairy surface. In addition to physical factors such as light, temperature, and humidity, the addition of an endogenous growth factor (GA_3) is also required for seed germination. It has been reported that in Arabidopsis, GA_3 release causes the seed coat to weaken by stimulating the expression of genes involved in cell expansion and modification **[18]**. Moreover, in their organogenesis studies on argan plants, Amghar et al. (2021) reported that GA_3 increased the germination frequency and that supplementing MS media with 1.0 mg L^{-1} BAP with 2.0 mg L^{-1} GA_3 increased the number of adventitious shoots formed **[19]**.

First, the seed germination frequency in our study was 6.6% in MSB media in the absence of growth regulators. Thus, different concentrations of the PGRs BAP and GA_3 were subsequently used to promote germination and increase the germination frequency. The best result (27.22%) was obtained in MSB media supplemented with 1.0 mg L^{-1} BAP and 1.0 mg L^{-1} GA_3. This study revealed that GA_3 ensures germination by breaking dormancy in the seeds while the hormone BAP increases shoot length. Since it is difficult to germinate the seed of Helichrysum species, micropropagation studies have generally been performed on aerial parts **[11, 12]**. These results demonstrate that seed sterilization is applicable under certain physical conditions and that germination is possible using different plant growth regulators (PGRs), such as BAP and GA_3.

Second, HAE shoot development was optimal in MSB media supplemented with 5.0 mg L^{-1} Kin and 0.5 mg L^{-1} IAA (60%). The experiment was stopped at the optimum concentration and that further testing might be needed to fully explore the dose-

response relationship, and to determine if higher concentrations might be even more effective. According to **[16],** supplementing MS media with the PGRs Kin and IAA positively affects height as well as shoot formation **[11]**. On the other hand, **[20]** in their micropropagation study on H. italicum, reported that the highest percentage of shoot regeneration occurred in DKW media supplemented with 5 µM Kin **[21]**. Optimized an in vitro clonal propagation method from H. umbraculigerum leaves and found that shoots were produced most efficiently on MS media containing a specific concentration of TDZ **[15]**. **[2]** reported that the highest shoot formation percentage from young H. italicum shoots occurred in MS media supplemented with 0.5 mg L^{-1} BAP, 1.0 mg L^{-1} GA_3, and 0.2 mg L^{-1} NAA, while the lowest percentage occurred in MS media without hormone supplementation. In another study, **[20]** micropropagated H. stoechas and H. italicum plants and reported that the number of shoots increased twofold in response to supplementation with BAP.

In our protocol, calli were formed via indirect organogenesis in medium enhanced with 1.0 mg L^{-1} BAP. The calli were moved to MSB media supplemented with various concentrations of the PGRs 2,4-D, TDZ, NAA, and picloram. Green, friable calli grew best on MSB media supplemented with 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} TDZ. Similarly, 81.3% green and friable calli of H. pallasii were obtained by using 1.0 mg L^{-1} TDZ **[22]**. The addition of different concentrations of different PGRs to calli growth media encourages root or shoot development to yield plantlets **[23]**.

The auxin PGRs IAA, IBA and NAA are the most frequently PGRs reported in the literature for rooting. Notably, both root induction and root elongation are extremely sensitive to auxin concentration **[24]**. Moreover, the addition of auxins positively affected root and adventitious shoot formation in our study. The ideal mixture for rooting in our study was MSB media enhanced with 1.0 mgL^{-1} IBA (77.8%). Although root development frequency varies depending on the plant species in terms of a hormonal or nonhormonal environments, it has generally been reported that the growth regulator IBA promotes rooting and increases shoot length **[25]**.

Conclusion

This is the first report of the successful micropropagation of HAE using plant tissue culture techniques. The protocol described here uses BAP, Kin, and IAA to induce the proliferation of multiple shoots on tissue explants, and BAP supplementation produced the most robust organogenic response via indirect organogenesis. Following shoot production, plantlets treated with IBA developed root systems. This micropropagation protocol, starting from seeds, provides an alternative to clonal propagation and contributes to the conservation of HAE, a rare and endemic plant species. Additionally, this study contributes to the development of in vitro methods for propagating other Helichrysum and rare plant species.

Supplementary Materials:

No Supplementary Materials.

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Informed Consent Statement:

Not applicable.

Data Availability Statement:

Data available upon request.

Conflicts of Interest:

The authors declare no conflict of interest.

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الإكثار الدقيق لنبات Helichrysum Arenarium (L.) Moench Subsp. تحت نوع Erzincanicum: نوع نباتي مستوطن.

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الخلاصة

طُوِّرت هذه الدراسة لوضع بروتوكول للإكثار الدقيق باستخدام أجزاء نباتية مُستنبتة من بذور نبات (Helichrysum arenarium (L.) Moench subsp. erzincanicum) بأستخدام تقنيات زراعة الأنسجة النباتية. في البداية، أُجريَ تكوين الأعضاء غير المباشر للبراعم باستخدام شتلات نبات (Helichrysum arenarium (L المزروعة في المختبر على وسط موراشيغ وسكوج (MS) ووسط غامبورغ B5 (يُختصر بـ MSB) المُدعَّم بـ 6-بنزيل أمينوبورين (BAP) والكينيتين (Kin) وحمض الإندول-3-أسيتيك (IAA). وقد لوحظ أعلى عدد من البراعم في الوسط المدعم بـ 1 ملغم/لتر من BAP. بعد ذلك، تم تلقيح البراعم في مزيج من وسط MSB مع 0.5-1.5 ملغم/لتر من حمض الإندول-3-أسيتيك وحمض الإندول-3-بيوتيريك (IBA) حيث أدى تركيز 1.0 ملغم/لتر من IBA (بنسبة 77.8%) إلى تحفيز أكبر عدد من الجذور. بالإضافة إلى ذلك، تم تحفيز تكوين الكالس باستخدام تراكيز مختلفة من الثيديازورون (TDZ)، 1.0، 2.0 ملغم/لتر) وحمض ألفا-نفثالين أسيتيك (NAA) ملغم/لتر) ولوحظت أعلى نسبة لتكوين الكالس في مزيج وسط MSB مع 2.0 ملغم/لتر من TDZ و1.0 ملغم/لتر من NAA. تقدم هذه الدراسة بروتوكولاً ناجحاً للإكثار الدقيق لنبات H. arenarium subsp. erzincanicum، وتدعم إكثاره في المختبر باستخدام أجزاء نباتية مُستنبتة من البذور. لذا، هدفت هذه الدراسة إلى تطوير طريقة فعّالة للإكثار الدقيق لهذا النوع الفرعي، باستخدام أجزاء نباتية مُستخلصة من بذور متوطنة في تركيا وتنمو فيها طبيعياً. يوفر بروتوكول الإكثار الدقيق هذا الذي يبدأ من البذور بديلاً للإكثار الخضري ويساهم في الحفاظ على نبات Helichrysum arenarium (L.) Moench subsp. erzincanicum وهو نوع نباتي نادر ومتوطن. إضافةً إلى ذلك، تُسهم هذه الدراسة في تطوير طرق الزراعة النسيجية لإكثار أنواع أخرى من نباتات Helichrysum وأنواع نباتية نادرة.

الكلمات المفتاحية: الأنواع المستوطنة، Helichrysum arenarium subsp. erzincanicum، حمض الجبرليك، الكينيتين، الإكثار الدقيق، البذور