

Micropropagation of Endemic Taxa of the Flora of Turkey: *Helichrysum arenarium* (L.) Moench subsp. *erzincanicum* P.H. Davis & Kupicha (Asteraceae)

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Abstract

This study developed a micropropagation protocol using 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 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 1 mg L⁻¹ BAP (61.2%). 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), wherein 1.0 mg L⁻¹ IBA (77.8%) recorded the maximum number of roots. Additionally, callus formation was achieved with different concentrations of thidiazuron (TDZ) (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 (80.2%) 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 *H. arenarium* subsp. *erzincanicum* and supports its *in vitro* propagation via explants grown from seed.

Introduction

Helichrysum arenarium (L.) Moench subsp. *erzincanicum* P.H. Davis & Kupicha (abbreviated as HAE) is endemic to Turkey, specifically distributed across the provinces of Kastamonu, Ağrı, Erzincan, and Kayseri. It is a perennial, herbaceous species endemic to Turkey, growing in its natural vegetation (Umaz & Umaz, 2020; Uz & Savalan, 2024). In Turkey, 30 *Helichrysum* species have been identified, 16 of which are endemic (Eroğlu, 2018). *Helichrysum* is also commonly known as golden grass, an everlasting or immortal flower (Czinner et al., 2000). *Helichrysum* has traditionally been used as a diuretic, cholagogue, hepatoprotective, bile-building, antithrombotic and capillary-sealing agent with effects in the gastrointestinal tract, liver and gallbladder (Liu et al., 2019).

Many *Helichrysum* species have been used not only in folk medicine but also for ornamental, cosmetic, and pharmaceutical purposes (Gradinaru et al., 2014; Pawelek, 2015). With bright yellow color, it is suitable for fresh and dry bouquet arrangements, as an insect repellent, essential oil and aromatic chemical glycosides for cosmetics, pharmaceutical and food industries are used (Dănăilă-Guidea et al., 2022). The essential oils and phytochemicals of *Helichrysum*, such as monoterpenoids, sesquiterpenes, triterpenoids, flavonoids, and pyrones, have important pharmaceutical properties (Jakupovic et al., 1986; Leonardi et al., 2018). 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, Figas et al. (2016) used the apical meristems of *H. arenarium* as explants and determined that the best plant growth regulator (PGR) combination for shoot regeneration

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 (Figas et al., 2016). Clasquin and Henry (2002) used shoots and stem nodes as explants for micropropagation of *H. arenarium* and found that more calli formed in Murashige and Skoog (MS) media supplemented with 1.0 mg L⁻¹ 2,4-D (Clasquin & Henry, 2002). Perrini et al. (2009) used the apical buds and axillary shoots of *Helichrysum italicum* subsp. *microphyllum* as explant sources (Perrini et al., 2009). Prinsloo and Meyer (2006) used the young leaves and axillary buds of *Helichrysum kraussii*, and calli formed in MS nutrient media supplemented with 1 mg L⁻¹ α-naphthaleneacetic acid (NAA) and 0.05 mg L⁻¹ KIN (Prinsloo & Meyer, 2006). Furthermore, Dimitrova and Nacheva (2018) reported the use of *H. italicum* axillary buds as explants for shoot regeneration (Prinsloo & Meyer, 2006); Botha et al. (2015) optimized an *in vitro* propagation protocol from *Helichrysum umbraculigerum* leaves (Botha et al., 2015); and Uz & Savalan (2024) used young *H. italicum* shoots for *in vitro* production (Uz & Savalan, 2024). However, to our knowledge, there are no prior reports on the micropropagation of endemic *H. arenarium* subsp. *erzincanicum*. Hence, this study aimed to develop an effective micropropagation method for this subspecies, using explants derived from seeds endemic to and naturally occurring in Turkey.

Materials and methods

Material

H. arenarium (L.) Moench subsp. *erzincanicum* Davis & Kupicha (Fig. 1) 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.

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, 70% ethanol was applied 30 min and then incubated at 42°C for 24 hours. The sterile seeds were rinsed for 5 times with sterile distilled water (Battal et al., 2019). After sterilization, the media was additionally supplemented with 1.0 mg L⁻¹ gibberellic acid (GA₃), 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 transferred to MS medium supplemented with 0.1, 0.5, or 1.0 mg L⁻¹ IAA or IBA for root formation.

Statistical analysis

The data were statistically analysed using SPSS for Windows (SPSS Inc., Chicago, IL, USA). Differences in means $\pm$ standard errors (SEs) were evaluated for significance using Duncan's one-way analysis of variance (ANOVA) at the $P < 0.05$ level.

Results

Seed germination rate

The effects of the PGRs; BAP and GA₃ on the germination of *H. arenarium* subsp. *erzincanicum* seeds on MSB medium are shown in Table 1. The highest germination rate (27.22%) was obtained in MSB media supplemented with 1.0 mg L⁻¹ GA₃ and 1.0 mg L⁻¹ BAP.

Table 1
Effects of the plant growth regulators BAP and GA₃ on the seed germination rate of *H. arenarium* subsp. *erzincanicum* in MSB media

Concentration of PGRs		Germination rate (%)
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

Different letters within the same column indicate a significant difference between means at the 0.05 level, and (±) denotes the standard error.

Shoot proliferation

The sterile plantlets obtained from the seeds 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 best shoot regeneration was observed MSB media supplemented with 1.0 mg L⁻¹ BAP (61.2%, $P < 0.05$).

Table 2
Effects of different concentrations of BAP in MSB media on *H. arenarium* subsp. *erzincanicum* shoot formation 8 weeks after culture initiation

Plant growth regulator (mg L ⁻¹)	Rate of shoot formation from the explants (%)
BAP	
0.5	19.8 ± 1.6 ^c
1.0	61.2 ± 4.2^a
2.0	38.03 ± 2.8 ^b

Different letters in the same column indicate a significant difference between means at the 0.05 level, and (±) indicates the standard error.

The shoots obtained from media supplemented with 1.0 mg L⁻¹ BAP were transferred to MSB media supplemented with different combinations of KIN and IAA (Figas et al., 2016). By the end of the 30th day, the plantlets had grown in height (Fig. 2).

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 highest percentage of shoot formation, reaching 60% (Table 3).

Table 3
Response of shoot formation of HAE on MSB media supplemented with different concentrations of KIN and IAA

PGR combination (mg L ⁻¹)		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 calli formation rates (p < 0.05). The highest rate of callus formation (80.2%) was obtained with MSB media supplemented with 2.0 mg L⁻¹ TDZ and 1.0 mg L⁻¹ NAA (Fig. 3).

Table 4
Effects of callus growth of HAE on MSB media with different concentrations of 2,4-D, TDZ, NAA, and picloram

PGR(s)		Callus structure and colour	Callus formation rate (%)
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	Brown, 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	Green, friable	80.2 ± 16.8^a

Different letters in the same column indicate a significant difference between means at the 0.05 level, and (±) indicates the standard error.

Rooting

The elongated shoots were subsequently transferred to MSB media supplemented with 0.5, 1.0, or 1.5 mg L⁻¹ IBA or IAA for rooting, and roots developed within four weeks of culture. The highest percentage (77.8%) of roots were found in the 1.0 mg L⁻¹ IBA treatment group, in which new adventitious shoots formed (Figs. 4 and 5).

Discussion

The sterilization of the *H. arenarium* subsp. *erzincanicum* seeds was challenging due to their small size and hairy structure. Battal et al. (2019) reported the sterilization method that was successfully applied in this study (Battal et al., 2019). 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 (GA₃) is also required for seed germination. It

has been reported that in *Arabidopsis*, GA₃ release causes the seed coat to weaken by stimulating the expression of genes involved in cell expansion and modification (Gupta & Chakrabarty, 2013). Moreover, in their organogenesis studies on argan plants, Amghar et al. (2021) reported that GA₃ increased the germination rate and that supplementing MS media with 1.0 mg L⁻¹ BAP with 2.0 mg L⁻¹ GA₃ increased the number of adventitious shoots formed (Amghar et al., 2021).

First, the seed germination rate in our study was 6.6% in MSB media in the absence of growth regulators. Thus, different concentrations of the PGRs BAP and GA₃ were subsequently used to promote germination and increase the germination rate. The best result (27.22%) was obtained in MS/B5 media supplemented with 1.0 mg L⁻¹ BAP and 1.0 mg L⁻¹ GA₃. This study revealed that GA₃ 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 (Clasquin & Henry, 2002; Figas et al., 2016). 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 GA₃.

Second, *H. arenarium* subsp. *erzincanicum* 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 Figas et al. (2016), supplementing MS media with the PGRs KIN and IAA positively affects height as well as shoot formation (Figas et al., 2016). On the other hand, Dimitrova and Nacheva (2018), in their micropropagation study on *H. italicum*, reported that the highest percentage of shoot regeneration occurred in DKW media supplemented with 5 µM KIN (Dimitrova & Nacheva, 2018). Botha et al. (2015) optimized an *in vitro* propagation protocol from *H. umbraculigerum* leaves and found that shoots were produced most efficiently on MS media containing a specific concentration of TDZ (Botha et al., 2015). Uz & Savalan (2024) 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 GA₃, and 0.2 mg L⁻¹ NAA, while the lowest percentage occurred in MS media without hormone supplementation (Uz & Savalan, 2024). In another study, Giovannini et al. (2003) micropropagated *H. stoechas* and *H. italicum* plants and reported that the number of shoots increased twofold in response to supplementation with BAP (Giovannini et al., 2001).

In our protocol, calli were formed via indirect organogenesis in media supplemented with 1.0 mg L⁻¹ BAP. The calli were subsequently transferred to MSB media supplemented with different 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 (Yurteri et al., 2021). The addition of different concentrations of different PGRs to calli growth media encourages root or shoot development to yield plantlets (Evans et al., 1981).

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 (Koca, 2015). Moreover, the addition of auxins positively affected root and adventitious shoot formation in our study. The ideal mixture for rooting in our study was MS/B5 media supplemented with 1.0 mg L^{-1} IBA (77.8%). Although the rooting rate 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 (Yurteri et al., 2021).

Conclusion

This is the first report of the successful micropropagation of *H. arenarium* subsp. *erzincanicum* 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 provides an alternative to seed propagation and contributes to the conservation of *H. arenarium* subsp. *erzincanicum*, 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.

Abbreviations

Gibberellic acid (GA₃)

Indole-3-acetic acid (IAA)

Murashige and Skoog (MS)

6-benzylaminopurine (BAP)

N6-furfuryladenine (kinetin (KIN))

Thidiazuron (TDZ)

α-naphthaleneacetic acid (NAA)

Plant growth regulator (PGR)

Sodium hypochlorite (SH)

Declarations

Author contribution statement

G.A: Data curation, Formal analysis, Methodology, Validation, Visualization, Writing – review & editing.
Ş.Ö: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft.

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Data availability

All data produced or analyzed during this study are included in this article. Data will be available upon request from the corresponding author.

Conflict of interest The authors have declared that they have no conflict of interest.

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Figures



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.

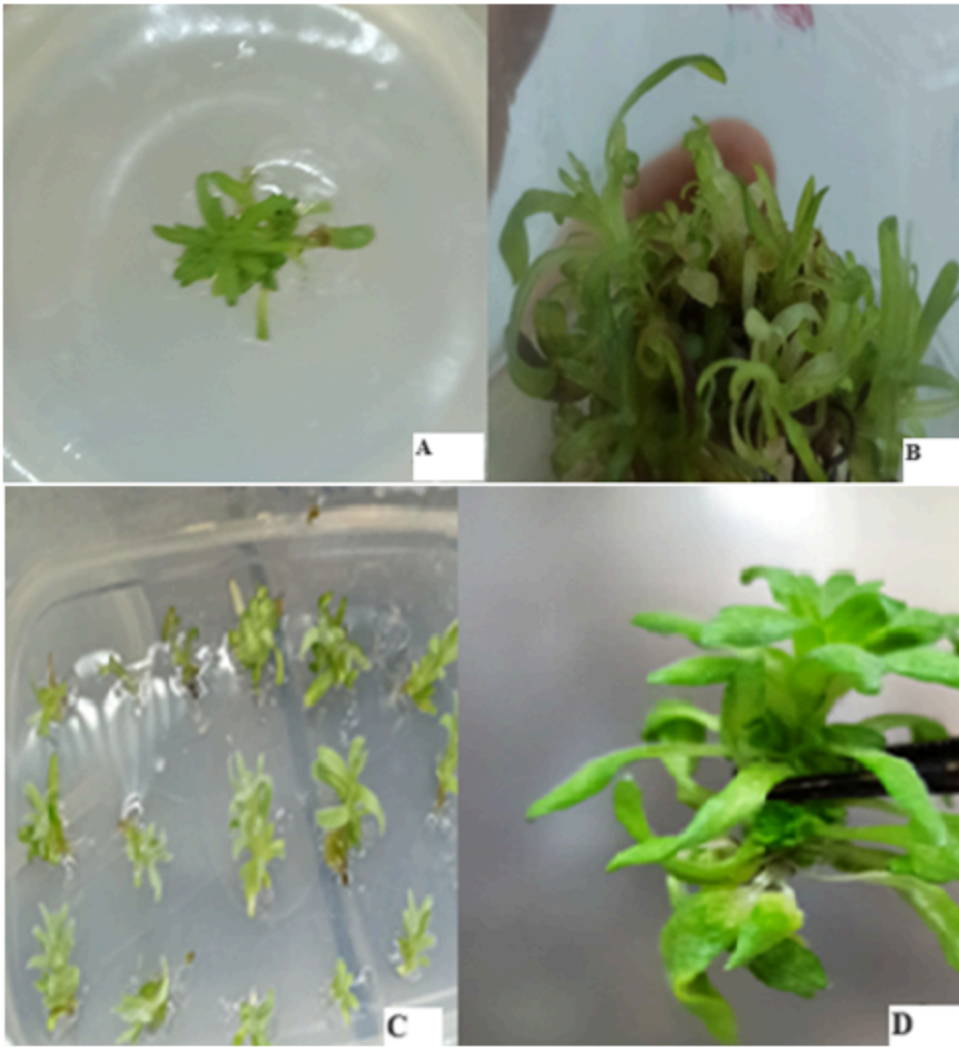


Figure 2

Shoot regeneration (A) on the first day after placing in MSB media supplemented with 1 mg L^{-1} BAP and (B) transfer of the plantlets after 8 weeks. (C) Images of plantlets transferred from media supplemented 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.

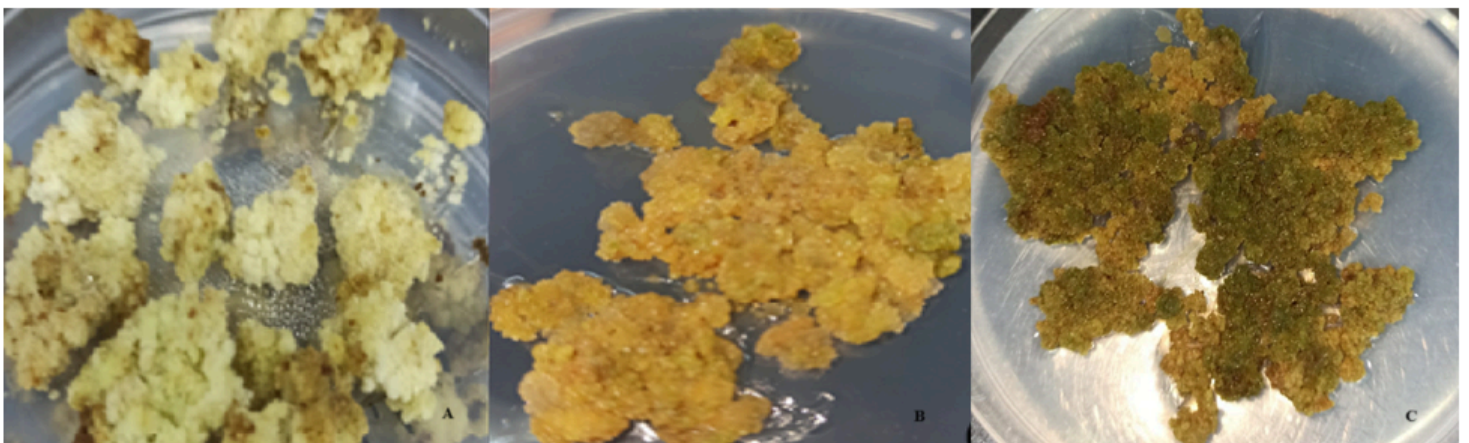


Figure 3

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.

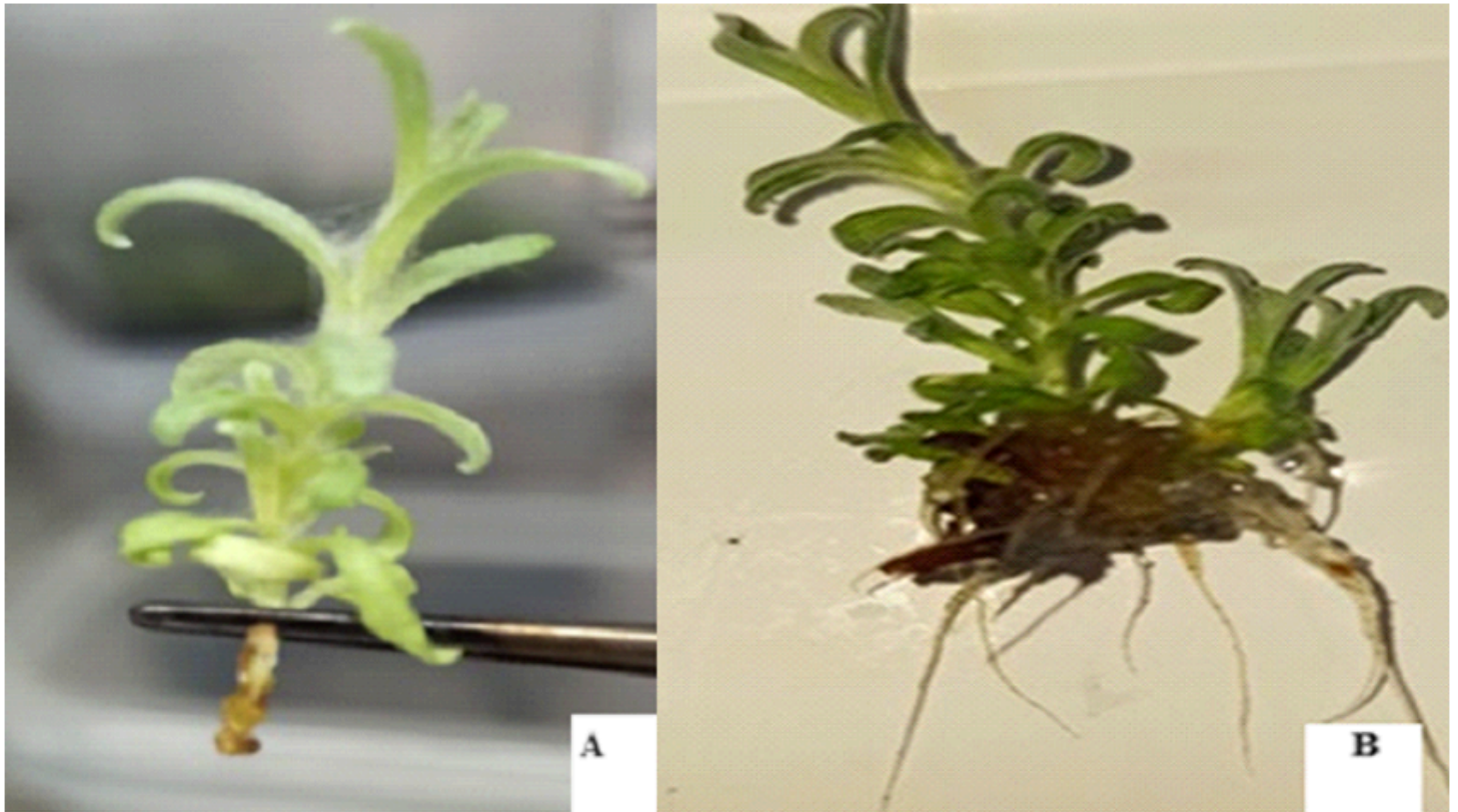


Figure 4

Representative images of plantlets transferred to media supplemented with 1.0 mg L⁻¹ IBA for rooting (A) on the first day and (B) after 30 days.

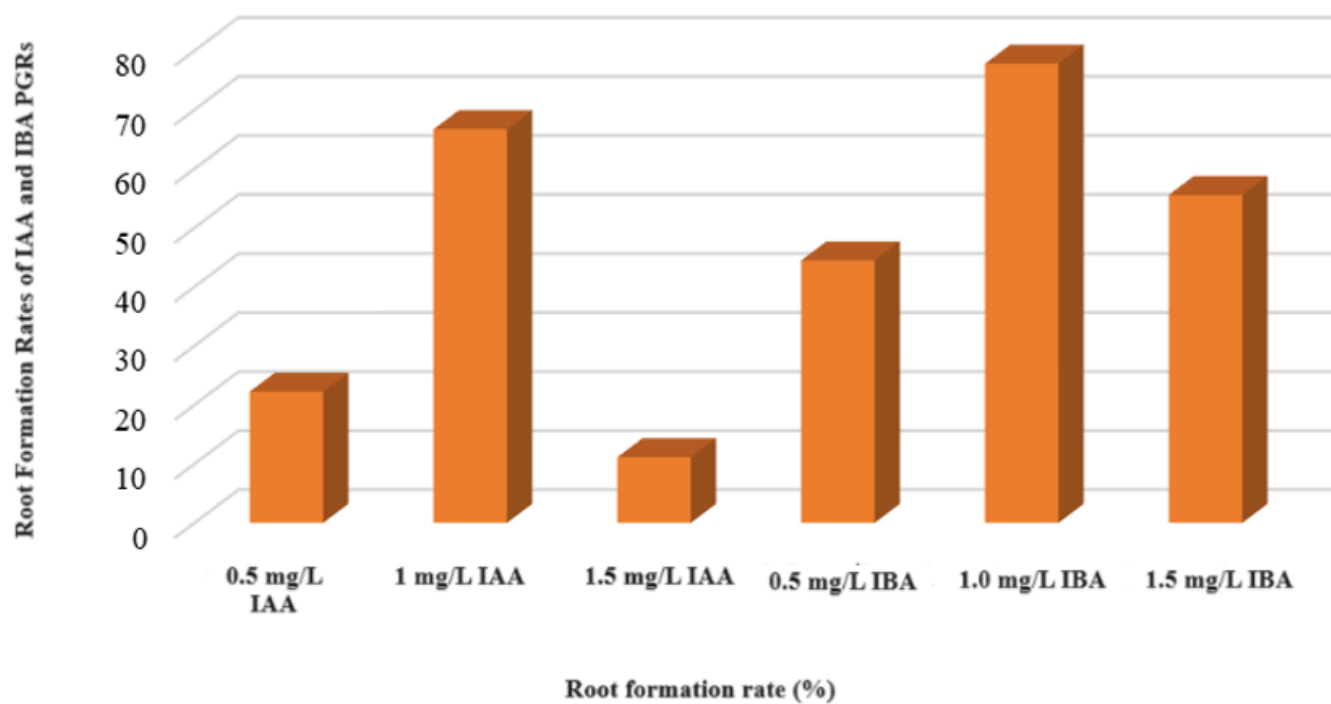


Figure 5

Root formation rates (%) after 30 days.