

***In vitro* direct organogenesis of the medicinal single-mountain local prioritized vulnerable Greek endemic *Achillea occulta* under different medium variants**

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Abstract

Achillea species are of high medicinal value based on their use in traditional medicine and their content in secondary metabolites with potential application in dermatology and cosmetic industry. *Achillea occulta* is a single-mountain local prioritized vulnerable Greek endemic encountering propagation problems through seeds and root division. *In vitro* propagation can be used for the rapid and mass production of clonal plants complying with phytochemical and sanitary quality criteria. In this study, several culture medium variants were tested including the effect of different indole-3-butyric acid (IBA) concentrations with 6-benzyladenine (BA), different cytokinin types, agar concentrations, and zeatin (ZT) sterilization methods on *in vitro* shoot proliferation, as well as the effect of different auxin types and concentrations on *in vitro* rooting and *ex vitro* survival rate of shoot-tip explants. The results showed BA was the most preferable cytokinin type as evidenced by all proliferation attributes and autoclaved ZT provided longer shoots. Therefore, the two more promising and cost-effective treatments for proliferation were 2.2 μM BA + 0.25 μM IBA or 4.4 μM BA + 0.5 μM IBA after 21 days of culture on modified Murashige-Skoog (MS) medium ($\times 2$ Fe) enriched with 20 g L^{-1} sucrose and 6-7 g L^{-1} Agar (90-93.8% shoot formation, 2.7-2.9 shoots 0.4-0.6 cm long, 2.5-2.6 proliferation rates). Explants rooted better after 21 days of culture on plain MS medium with 2 μM IBA or 1 μM α -naphthalene acetic acid (NAA) (13.3-20% rooting, 1-1.5 roots 1.8-2 cm long). NAA gave more roots, IBA longer roots, while both auxins exhibited highest rooting (13.33-20%) and *ex vitro* survival rates (50-66.7%).

Keywords: auxins; cytokinins; *ex situ* conservation; gelling agents; Greek yarrow; micropropagation; sterilization methods

Introduction

Plant species that belong to the genus *Achillea* (Asteraceae) have been reported to be of high medicinal value based on their use in traditional medicine as a natural remedy for the healing of several ailments including headache, pains, wounds, flatulence, bleedings, inflammation, dyspepsia, and spasmodic diseases

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(Mohammadhosseini *et al.*, 2017). The medicinal properties within *Achillea* species are attributed to their content in a wide variety of secondary metabolites such as flavonoids, phenolic acids, terpenes, phyosterols, organic acids, fatty acids, and alcohols with potential use in dermatology and cosmetic industry as rich sources of active phytochemicals (Strzpek-Gomólka *et al.*, 2021). More than 130 *Achillea* species are wild perennial herbs, the majority distributed mainly in Northeastern Europe and Northwestern Asia, exhibiting antimicrobial, antioxidant, and anti-inflammatory action (Stojanovic *et al.*, 2005).

According to Constantinidis and Kalpoutzakis (2009), *Achillea occulta* is a single-mountain local endemic of the Greek flora with conservation priority as a threatened one due to fires and overgrazing. Based on IUCN criteria (2001), this species has been characterized as 'vulnerable' (Constantinidis and Kalpoutzakis, 2009) and is not protected by the Hellenic Presidential Decree 67/81 (Dimopoulos *et al.*, 2016). The natural habitat of this plant includes cliffs, rocks, walls, ravines, and boulders in Mt Koulochera and Komboriza, SE Peloponnese, Greece, at 700-1.126 m (Constantinidis and Kalpoutzakis, 2009).

Seed germination and asexual root division are traditional propagation methods used for *Achillea* spp. also commonly known as yarrows but in a very restricting time period i.e., a particular month of the year (Chaturvedi *et al.*, 2007). Mass production of pathogen-free and genetically uniform plant material of yarrow on a commercial scale both qualitatively and quantitatively, and throughout the whole year can be achieved through plant tissue culture strategies including micropropagation for standardized formulations in the chemical constituents of the plants and their parts, fulfilling the ever-increasing demand of uniform plant-based medicines warrants (Chaturvedi *et al.*, 2007). New methods derived from advances in biotechnology could contribute more efficiently to the conservation of plant diversity and its subsequent evaluation process (Manole-Păunescu, 2014). In addition, new methodological biotechnology tools can reinforce *in situ* measures and *ex situ* classical strategies related to the collection, multiplication, and conservation of plant biodiversity (Holobiuc *et al.*, 2018). Up to now, there is a limited number of research studies regarding *in vitro* regeneration of *Achillea* species including calli and cell suspension cultures of *A. millefolium* (Figueiredo and Pais, 1991), *in vitro* propagation of *A. ptarmica* (Cellarova *et al.*, 1982), micropropagation of *A. asplenifolia* (Wawrosch *et al.*, 1994) and *A. occulta* (Grigoriadou *et al.*, 2011), callus induction of *A. biebersteinii* from leaf and petiole explants (Kahrizi and Kakaei, 2010).

This study was undertaken with the objective to develop an efficient *in vitro* protocol that would maintain and propagate *A. occulta*, an important species by testing several factors in an attempt to optimize the explants response. Several parameters were taken into consideration for the *in vitro* shoot proliferation stage of *A. occulta* including the effect of (1) different indole-3-butyric acid (IBA) concentrations combined with 6-benzyladenine (BA), (2) different concentrations of the gelling agent "agar", (3) different cytokinin types and (4) different sterilization methods of the cytokinin "zeatin (ZT)" either autoclave-sterilized (no filter used) or filter-sterilized without undergo autoclaving, applied to the culture medium alone and in combination with different BA concentrations. In the following stages, different auxin types and concentrations were tested in order to determine the ideal auxin type and its optimum concentration for maximal *in vitro* rooting performance and better adaptation of the rooted microshoots to *ex vitro* greenhouse conditions through a gradual acclimatization and hardening process.

Materials and Methods

Plant material and culture conditions

Shoot-tip explants, 0.5 cm long, derived from previous *in vitro* cultures sub-cultured every four weeks on MS (Murashige and Skoog, 1962) (Duchefa Biochemie, The Netherlands) basal medium containing 1.32 μM 6-benzyladenine (BA) (Duchefa Biochemie, The Netherlands), 0.15 μM indole-3-butyric acid

(IBA)(Duchefa Biochemie, The Netherlands), 20 g L⁻¹ sucrose (Duchefa Biochemie, The Netherlands) and 6.5 g L⁻¹ agar (Sigma-Aldrich) were used as the experimental plant material in all five experiments conducted, the first four related to *in vitro* shoot proliferation, and the fifth and last one regarding *in vitro* rooting. The pH value of the culture media was adjusted to 5.8 before adding the gelling agent and prior to autoclaving at 121 °C for 20 min. The explants were transferred into Magenta™ vessels (Baby food jars, autoclavable, reusable, 62.4 × 95.8 mm, size: 200 mL, Sigma-Aldrich, owned by Merck KGaA, Darmstadt, Germany), each containing 35 mL of the culture media as described below. Magenta™ B-caps were used for covering the vessels. The cultures were maintained in a growth chamber under 16h light/ 8h darkness photoperiod regime, light intensity of 40 μmol m⁻² s⁻¹ supplied by cool white fluorescent lamps, and temperature of 22 ± 1 °C. The same controlled environmental conditions (photoperiod, illumination, temperature) were followed for all experiments carried out and described below during the *in vitro* cultures of *A. occulta* explants inside the growth chamber.

In vitro shoot proliferation

Different IBA concentrations combined with BA

Five different concentrations of the auxin IBA (0, 0.5, 1.25, 2.5 and 5 μM) were tested. The basal culture medium used was the modified MS [double amount of Fe (x2 Fe)] enriched with 20 g L⁻¹ sucrose, 4.4 μM BA and solidified with 6 g L⁻¹ Plant Agar (Duchefa Biochemie). After 21 days of culture, the following measurements were recorded: percentage of shoot formation (%), number of shoots per explant, shoot length (cm) and proliferation rate. The experiment included 5 treatments with 16 repetitions (explants)/ treatment (4 explants/ vessel x 4 vessels/ treatment).

Different agar concentrations

Three different concentrations of the gelling agent “agar” (Sigma-Aldrich) (6, 7 and 8 g L⁻¹) were tested. The culture medium used was the modified MS (x2 Fe) enriched with 20 g L⁻¹ sucrose, 2.2 μM BA and 0.25 μM IBA. After 21 days of culture, the following measurements were recorded: percentage of shoot formation (%), number of shoots per explant, shoot length (cm) and proliferation rate. The experiment included 3 treatments with 20 repetitions/ treatment (4 explants/ vessel x 5 vessels/ treatment).

Different cytokinin types

Four different cytokinin types, i.e. BA, 2-isopentenyl-adenine (2-ip), zeatin (ZT) and kinetin (KIN), each applied in a 4.4 μM concentration were tested. The culture medium used was the modified MS (x2 Fe) enriched with 20 g L⁻¹ sucrose, 0.44 μM IBA and solidified with 7 g L⁻¹ Plant Agar. After 21 days of culture, the following data were recorded: percentage of shoot formation (%), number of shoots per explant, shoot length (cm) and proliferation rate. The experiment included 5 treatments with 20 repetitions/ treatment (4 explants/ vessel x 5 vessels/ treatment).

ZT application method and BA concentration

ZT applied either autoclave sterilized (no-filter used) or filter-sterilized without autoclaving in a concentration of 4.4 μM. The individual effect of BA applied at 4.4 μM as well as the combined effect of 4.4 μM ZT (non-filtered, filtered) with 4.4 μM BA were tested. Therefore, the experiment included five treatments, i.e. 4.4 μM BA, 4.4 μM ZT (non-filtered), 4.4 μM ZT (filtered), 4.4 μM ZT (non-filtered) + 4.4 μM BA, and 4.4 μM ZT (filtered) + 4.4 μM BA. The culture medium used was the modified MS (x2 Fe) enriched with 20 g L⁻¹ sucrose, 0.44 μM IBA and solidified with 7 g L⁻¹ Plant Agar. After 21 days of culture, the following data were recorded: percentage of shoot formation (%), number of shoots per explant, shoot length (cm) and proliferation rate. The experiment included 5 treatments with 20 repetitions/ treatment (4 explants/ vessel x 5 vessels/ treatment).

In vitro rooting under different auxin types and concentrations

Two different auxin types, i.e., IBA and NAA each applied either alone in four different concentrations (0, 1, 2, 5 μM) or combined IBA + NAA in three different concentrations (1 + 1, 2 + 2, 5 + 5 μM) were tested. The culture medium used was the MS enriched with 20 g L⁻¹ sucrose and 6.5 g L⁻¹ Plant Agar. After 21 days, the following data were recorded: rooting percentage (%), root number/ rooted explant and root length (cm). The experiment included 10 treatments with 15 repetitions/ treatment (5 explants/ vessel x 3 vessels/ treatment).

Ex vitro acclimatization, hardening and survival of rooted microshoots

During early spring, rooted microshoots were rinsed with tap water to remove the adhering medium and planted in multi-hole propagation trays (100 mL volume per plantlet) containing a substrate mixture composed of peat (Terrahum) and perlite (1:1 v/v). The trays were transferred to a heated mist system in the greenhouse [at 18-21 °C base temperature, 15-25 °C air temperature and 70-85% relative humidity (RH)] for 30 days under reduced light intensity conditions (thermal curtains). Following that, the plants were successively transplanted in larger pots (0.33 L and 2.5 L) containing a peat moss (TS2): perlite: soil (2: 2: 1/2 v/v) substrate, and then placed in the greenhouse bench under increased light intensity and gradual RH decrease (5% per day) conditions. In early summer the plants transferred outside the greenhouse under a 50% shading net and automated sprinkling irrigation system.

Statistical analysis

The experimental layout was completely randomized. The means were subjected to analysis of variance (ANOVA) and compared using the Duncan multiple-range test ($p \leq 0.05$). The first, second and third *in vitro* shoot proliferation experiments were not multi-factorial and means were subjected to one-way ANOVA. The fourth shoot proliferation experiment was a 2×2 factorial one with two different application methods of ZT (non-filtered-autoclave-sterilized, filtered-sterilized without autoclaving) and two BA concentrations (0 and 4.4 μM). The main effect of factors (ZT application method, BA concentration) and their interaction was determined by General Linear Model/ 2-way ANOVA). The rooting experiment was a 3×4×4 factorial one with three auxin types (IBA, NAA, IBA + NAA), four IBA concentrations (0, 1, 2 and 5 μM), and four NAA concentrations (0, 1, 2 and 5 μM). The main effect of factors (auxin type, IBA concentration, NAA concentration) and their interactions were determined by General Linear Model/ 3-way ANOVA.

Results

In vitro shoot proliferation

Different IBA concentrations combined with BA

Shoot formation percentages (81.25-93.75%), shoot numbers per explant (2.25-2.69) and shoot lengths (0.62-0.74 cm) did not differ significantly regardless of IBA concentration (0-5 μM) combined with 4.4 μM BA (Table 1; Figures 1A-F). However, proliferation rate was significantly increased (2.52) in the combination treatment 4.4 μM BA + 0.5 μM IBA (Table 1).

Table 1. *In vitro* shoot proliferation of *A. occulta* after 21 days of culture in modified MS medium (x2 Fe) with 20 g L⁻¹ sucrose and 6 g L⁻¹ agar (pH: 5.8) under different IBA concentrations (0-5 μM) combined with 4.4 μM BA

Treatments	Shoot formation (%)	Shoot number/explant	Shoot length (cm)	Proliferation rate
4.4 μM BA + 0 μM IBA	81.25 ± 6.25 a	2.31 ± 0.22 a	0.74 ± 0.04 a	1.88 ± 0.18 b
4.4 μM BA + 0.5 μM IBA	93.75 ± 6.25 a	2.69 ± 0.25 a	0.62 ± 0.03 a	2.52 ± 0.24 a
4.4 μM BA + 1.25 μM IBA	81.25 ± 18.75 a	2.50 ± 0.24 a	0.73 ± 0.04 a	2.03 ± 0.20 ab
4.4 μM BA + 2.5 μM IBA	81.25 ± 11.97 a	2.25 ± 0.19 a	0.70 ± 0.04 a	1.83 ± 0.16 b
4.4 μM BA + 5 μM IBA	81.25 ± 11.97 a	2.38 ± 0.26 a	0.65 ± 0.04 a	1.93 ± 0.21 b
p-values (one-way ANOVA)	0.924 ns	0.700 ns	0.185 ns	0.049*

Means (n=16) ± standard error (S.E.) with different letters in a column for each proliferation parameter denote significant differences among treatments (Duncan test, p ≤ 0.05). ns p > 0.05, * p ≤ 0.05.

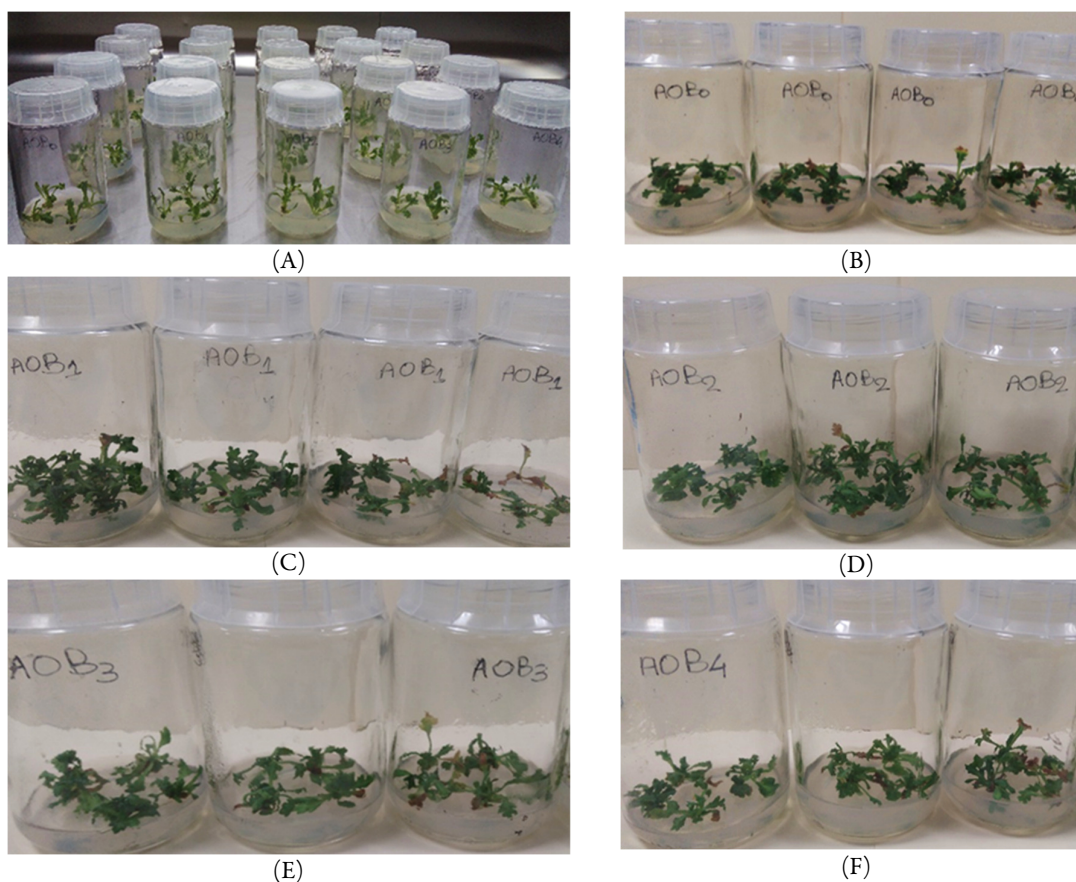


Figure 1. *In vitro* shoot proliferation of *A. occulta* after 21 days of culture in modified MS medium (x2 Fe) with 20 g L⁻¹ sucrose and 6 g L⁻¹ agar (pH: 5.8) under different IBA + BA concentrations; (A) All treatments; (B) 4.4 μM BA (IBA-free); (C) 4.4 μM BA + 0.5 μM IBA; (D) 4.4 μM BA + 1.25 μM IBA; (E) 4.4 μM BA + 2.5 μM IBA; (F) 4.4 μM BA + 5 μM IBA

Different agar concentrations

The increase in agar concentration (6-8 g L⁻¹) did not influence substantially the number of shoots/explant (2.20-2.90) (Table 2; Figures 2A-C). On the other hand, shoot lengths were significantly higher (0.40-0.42 cm) when 6 and 7 g L⁻¹ agar were added to the culture medium, as compared with the shortest microshoots (0.36 cm) recorded with 8 g L⁻¹ agar. The intermediate agar concentration of 7 g L⁻¹ exhibited the highest

proliferation rate of 2.61 being significantly different from those (1.43-1.84) obtained in the other two agar marginal concentrations. Shoot formation reached the highest value (90%) with 7 g L⁻¹ agar followed by 6 g L⁻¹ agar with non-significant difference. The further increase in agar concentration from 7 to 8 g L⁻¹ agar led to a substantial decrease by 35% in shoot formation, from 90% to 65%, respectively. Non-significant was the difference in shoot formation percentages (80% and 65%) between the lowest (6 g L⁻¹) and highest (8 g L⁻¹) agar concentration (Table 2).

Table 2. *In vitro* shoot proliferation of *A. occulta* after 21 days of culture in modified MS medium (x2 Fe) with 2.2 μM 6-benzyladenine (BA), 0.25 μM indole-3-butyric acid (IBA), 20 g L⁻¹ sucrose and 6 g L⁻¹ agar (pH: 5.8) under different agar concentrations (6, 7 and 8 g L⁻¹)

Treatments	Shoot formation (%)	Shoot number/explant	Shoot length (cm)	Proliferation rate
6 g L ⁻¹ agar	80.00 ± 9.35 ab	2.30 ± 0.22 a	0.40 ± 0.01 a	1.84 ± 0.18 b
7 g L ⁻¹ agar	90.00 ± 6.12 a	2.90 ± 0.28 a	0.42 ± 0.01 a	2.61 ± 0.25 a
8 g L ⁻¹ agar	65.00 ± 10.00 b	2.20 ± 0.26 a	0.36 ± 0.01 b	1.43 ± 0.17 b
p-values (one-way ANOVA)	0.049*	0.117 ns	0.010**	0.000***

Means (n=20) ± standard error (S.E.) with different letters in a column for each proliferation parameter denote significant differences among treatments (Duncan test, p ≤ 0.05). ns p > 0.05, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.

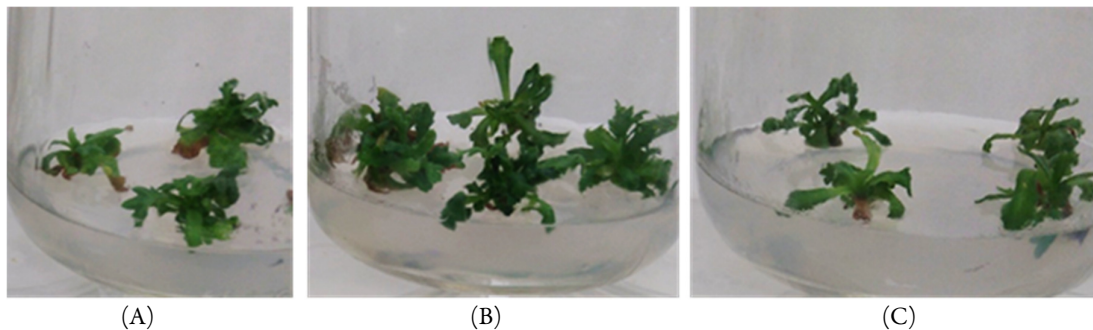


Figure 2. *In vitro* shoot proliferation of *A. occulta* after 21 days of culture in modified MS medium (x2 Fe) with 20 g L⁻¹ sucrose, 2.2 μM BA and 0.25 μM IBA (pH: 5.8) under different agar concentrations; (A) 6 g L⁻¹ agar, (B) 7 g L⁻¹ agar, and (C) 8 g L⁻¹ agar

Different cytokinin types

In the case of the control containing 0.44 μM IBA (cytokinin-free), only the 5% of the explants resulted in multiple shoot formation. Similar levels in higher shoot formation percentages (60-85%) were recorded in the other treatments containing different cytokinin types, differing statistically significant as compared with the control (5%) (Table 3; Figures 3A-E). All four culture media enriched with different cytokinin types (BA, 2-ip, ZT, KIN) led to a significant increase in shoot numbers (1.85-2.55) and proliferation rates (0.96-1.66) in relation to the control (1.05 shoots/ explant with a 0.49 proliferation rate). Between BA and KIN, BA was more beneficial in raising shoot number. Concerning shoot length, ZT was the preferred cytokinin type as compared to KIN, whereas the other three treatments i.e control, BA + IBA, and 2-ip + IBA exhibited similar non-significant values with either ZT + IBA or KIN + IBA. Among the four cytokinins, BA was the most effective in terms of shoot formation % than the other three cytokinins (2-ip, ZT, KIN) presenting non-significant differences. In particular, shoot number (2.55) and proliferation rate (1.66) were maximum under BA + IBA treatment, while shoot length was the largest (0.70 cm) in the combined effect ZT + IBA. The absolute highest value for shoot formation (85%) was achieved by adding BA + IBA to the culture medium. Therefore, taking simultaneously all shoot multiplication parameters into consideration, BA was the most effective cytokinin type (Table 3).

Table 3. *In vitro* shoot proliferation of *A. occulta* after 21 days of culture in modified MS medium (x2 Fe) with 20 g L⁻¹ sucrose and 7 g L⁻¹ Plant Agar (pH: 5.8) under different cytokinin types (BA, 2-ip, ZT, KIN) each applied at 4.4 μM in combination with 0.44 μM IBA

Treatments (μM)	Shoot formation (%)	Shoot number/explant	Shoot length (cm)	Proliferation rate
0.44 IBA (cytokinin-free)	5.00 ± 5.00 b	1.05 ± 0.05 c	0.46 ± 0.06 ab	0.49 ± 0.07 c
4.4 BA + 0.44 IBA	85.00 ± 10.00 a	2.55 ± 0.22 a	0.56 ± 0.05 ab	1.66 ± 0.18 a
4.4 2-ip + 0.44 IBA	75.00 ± 15.81 a	2.10 ± 0.20 ab	0.57 ± 0.05 ab	1.23 ± 0.14 b
4.4 ZT + 0.44 IBA	60.00 ± 15.00 a	2.00 ± 0.22 ab	0.70 ± 0.04 a	0.99 ± 0.13 b
4.4 KIN + 0.44 IBA	60.00 ± 15.00 a	1.85 ± 0.20 b	0.59 ± 0.04 b	0.96 ± 0.12 b
p-values (one-way ANOVA)	0.003**	0.000***	0.017*	0.000***

Means (n=20) ± standard error (S.E.) with different letters in a column for each proliferation parameter denote significant differences among treatments (Duncan test, p ≤ 0.05). * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.

**Figure 3.** *In vitro* shoot proliferation of *A. occulta* after 21 days of culture in modified MS medium (x2 Fe) with 20 g L⁻¹ sucrose, 0.44 μM IBA and 7 g L⁻¹ Plant Agar (pH: 5.8) under different cytokinin types (4.4 μM); (A) cytokinins-free; (B) BA; (C) 2-ip; (D) ZT; (E) KIN

ZT application method and BA concentration

BA, or ZT (autoclave sterilized or filter-sterilized without autoclaving) applied either alone or in combination with BA gave 60-85% shoot formation percentages and 2.00-2.55 shoots/ explant without significant differences. Shoot length was significantly higher (0.70 cm) in autoclave-sterilized ZT application, while proliferation rates were significantly higher when BA used alone (1.66 cm) or when filter-sterilized ZT combined with BA (1.51 cm) (Table 4).

Table 4. *In vitro* shoot proliferation of *A. occulta* after 21 days of culture in modified MS medium (x2 Fe) with 0.44 μM IBA, 20 g L⁻¹ sucrose, and 7 g L⁻¹ Plant Agar (pH: 5.8) under different ZT application methods (autoclave sterilized – no filter, filter-sterilized without autoclaving) combined with BA

Treatments (μM)	Shoot formation (%)	Shoot number/explant	Shoot length (cm)	Proliferation rate
4.4 BA	85.00 ± 10.00 a	2.55 ± 0.22 a	0.56 ± 0.05 b	1.66 ± 0.18 a
4.4 ZT (no-filter)	60.00 ± 15.00 a	2.00 ± 0.22 a	0.70 ± 0.04 a	0.99 ± 0.13 b
4.4 ZT (filter)	70.00 ± 9.35 a	2.10 ± 0.22 a	0.44 ± 0.02 c	1.40 ± 0.14 ab
4.4 BA + 4.4 ZT (no-filter)	80.00 ± 9.35 a	2.05 ± 0.15 a	0.49 ± 0.03 bc	1.40 ± 0.11 ab
4.4 BA + 4.4 ZT (filter)	70.00 ± 14.58 a	2.30 ± 0.24 a	0.53 ± 0.03 bc	1.51 ± 0.16 a
p-values (2-way ANOVA/ General Linear Model)				
ZT (non-filtered, filtered) (A)	0.910 ns	0.804 ns	0.000***	0.165 ns
BA Concentration (B)	0.315 ns	0.216 ns	0.179 ns	0.022*
(A)*(B)	0.315 ns	0.804 ns	0.000***	0.118 ns

Means (n=20) ± standard error (S.E.) with different letters in a column for each proliferation parameter denote significant differences among treatments (Duncan test, p ≤ 0.05). ns p > 0.05, * p ≤ 0.05, *** p ≤ 0.001.

In vitro rooting under different auxin types and concentrations, and *ex vitro* acclimatization, hardening and survival of rooted microshoots

Root formation occurred in the control (auxin-free) (6.67%) and by adding either 2 μM IBA (20%) or 1-2 μM NAA (6.67-13.33%) to the medium. No rooting occurred under the combined effect of IBA + NAA irrespective their concentrations, the highest NAA (5 μM), and the two marginal IBA concentrations (1 and 5 μM). The percentage of rooted microshoots was significantly higher (20%) with 2 μM IBA, followed by 1 μM NAA (13.33%) without differing substantially. NAA applied at 2 μM gave the highest root number (3 roots/rooted explant) while 2 μM IBA gave the longest roots (2 cm). Among the 10 rooting *in vitro* culture media tested, only four resulted in root formation i.e., control (auxins-free), 2 μM IBA, 1 μM , and 2 μM NAA. Rooted microshoots derived from medium devoid of auxins as well as from medium enriched with 2 μM NAA did not manage to acclimatize to the *ex vitro* conditions after a 30-day period in the greenhouse mist. However, *in vitro* plantlets that rooted on media supplemented with either 2 μM IBA or 1 μM NAA exhibited 66.67% and 50% survival rates, respectively, being non-statistically significant (Table 5; Figures 4A-D).

Table 5. *In vitro* rooting of *A. occulta* after 21 days of culture in MS medium with 20 g L⁻¹ sucrose and 6.5 g L⁻¹ Plant Agar (pH: 5.8) under different auxin types (IBA, NAA, IBA+NAA) and concentrations (1, 2, 5, 1+1, 2+2, 5+5 μM), and *ex vitro* survival rate of rooted microshoots

Treatments (μM)	Rooting (%)	Root number/ rooted explant	Root length (cm)	<i>Ex vitro</i> survival rate (%)
0 IBA + 0 NAA	6.67 \pm 6.67 bc	1.00 \pm 0.00 c	1.50 \pm 0.00 b	0.00 \pm 0.00 b
1 IBA + 0 NAA	0.00 \pm 0.00 c	0.00 \pm 0.00 d	0.00 \pm 0.00 e	0.00 \pm 0.00 b
2 IBA + 0 NAA	20.00 \pm 0.00 a	1.00 \pm 0.00 c	2.00 \pm 0.05 a	66.67 \pm 33.33 a
5 IBA + 0 NAA	0.00 \pm 0.00 d	0.00 \pm 0.00 d	0.00 \pm 0.00 e	0.00 \pm 0.00 b
0 IBA + 1 NAA	13.33 \pm 6.67 ab	1.50 \pm 0.05 b	1.38 \pm 0.01 c	50.00 \pm 28.87 a
0 IBA + 2 NAA	6.67 \pm 6.67 bc	3.00 \pm 0.00 a	0.83 \pm 0.00 d	0.00 \pm 0.00 b
0 IBA + 5 NAA	0.00 \pm 0.00 c	0.00 \pm 0.00 d	0.00 \pm 0.00 e	0.00 \pm 0.00 b
1 IBA + 1 NAA	0.00 \pm 0.00 c	0.00 \pm 0.00 d	0.00 \pm 0.00 e	0.00 \pm 0.00 b
2 IBA + 2 NAA	0.00 \pm 0.00 c	0.00 \pm 0.00 d	0.00 \pm 0.00 e	0.00 \pm 0.00 b
5 IBA + 5 NAA	0.00 \pm 0.00 c	0.00 \pm 0.00 d	0.00 \pm 0.00 e	0.00 \pm 0.00 b
p-values (3-way ANOVA/ General Linear Model)				
Auxin type (A)	1.000 ns	1.000 ns	1.000 ns	1.000 ns
IBA concentration (B)	0.009**	0.000***	0.000***	0.002**
NAA concentration (C)	0.216 ns	0.000***	0.000***	0.022*
(A)*(B)	0.078 ns	0.004**	0.000***	0.073 ns
(A)*(C)	0.519 ns	0.000***	0.000***	0.340 ns
(B)*(C)	0.051 ns	0.000***	0.000***	0.013*
(A)*(B)*(C)	0.015*	0.000***	0.000***	0.002**

Means (n=15) \pm standard error (S.E.) with different letters in a column for each parameter denote significant differences among treatments (Duncan test, $p \leq 0.05$). ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

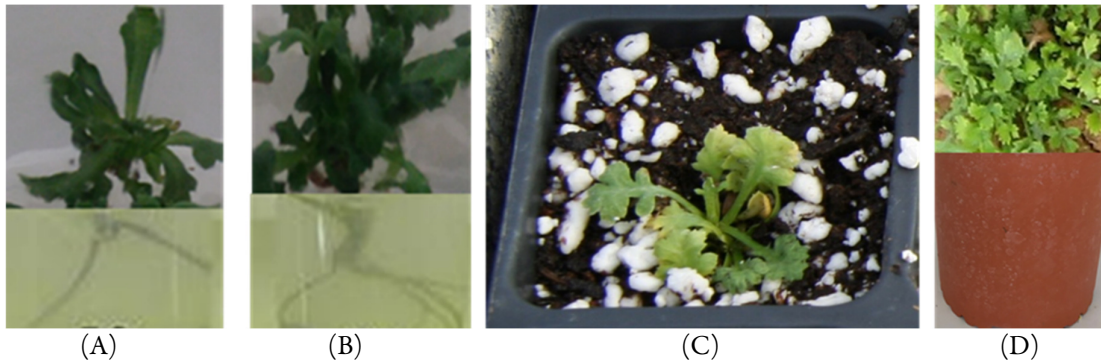


Figure 4. *In vitro* rooting and *ex vitro* acclimatization of *A. occulta*; (A) Rooted microshoot in medium enriched with 1 μM NAA; (B) Rooted microshoot in medium enriched with 2 μM IBA; (C) *Ex vitro* acclimatized plantlet within 0.33 L pot in the greenhouse bench; (D) Vegetative growth of acclimatized plantlet within a 2.5 L pot outside the greenhouse

Discussion

In vitro shoot proliferation

Different IBA concentrations combined with BA

The concentration of macronutrients in the MS culture medium is 3- to 4-fold higher than in other media tested (Murashige and Skoog, 1962). In this study, the MS medium was used as the basal one but modified on the concentration of chelate Fe which was doubled. According to Monteiro *et al.* (2000), there are several factors in tissue culture systems such as genotype, temperature, pH, and plant growth regulators (PGRs), among others that affect the uptake, transport or requirement of Fe, leading to decreased leaf chlorophyll content and phenomena of chlorosis. The reason for the use of the chelate form and double concentration of Fe in the MS basal medium [FeNaEDTA (x2)] in this study with *A. occulta* can be attributed to the fact that Fe becomes less exchangeable and available to plants as compared with the chelate form of Fe, especially under the lower temperatures used for incubation of tissue cultures inside growth chambers (Grigoriadou *et al.*, 2011). The use of different categories of PGRs mainly cytokinins and auxins, different types of cytokinins (i.e., BA, 2-ip, ZT, KIN) or auxins (IBA, NAA, IAA, 2,4-D) and their ratio as incorporated into the culture medium play a critical role in the established type of culture (Păunescu, 2009). In the present study, 4.4 μM BA combined with the lowest applied IBA concentration of 0.5 μM proved to be the most effective cytokinin/auxin ratio (9:1) regarding proliferation rate (2.52), while BA along with higher IBA concentrations (1.25-5 μM) gave non-significant proliferation rates as compared with the control (IBA-free). In consistency with our findings, shoot proliferation rate is reported to be enhanced under combinations of higher cytokinin to lower auxin concentrations (Biswas *et al.*, 2011), possibly due to higher level of degradative metabolites in tissues induced by higher auxin concentration, which ceases the regeneration ability of tissues (Baker and Wetzstein, 2004). Our results are in agreement with those reported by Grigoriadou *et al.* (2011), where the addition of 5 μM BA + 2.5 μM IBA (cytokinin/auxin, 2:1 ratio) to the modified MS medium (x 2Fe) was the most appropriate proliferation treatment for *A. occulta* (3.5 new shoots/ explant 0.93 cm long). The *in vitro* culture of *A. biebersteinii* was best promoted by adding 8.8 μM BA to the MS basal medium (Ghasempour *et al.*, 2012).

Different agar concentrations

In this study, the percentage of explants with multiple shoot formation and the length of *A. occulta* microshoots was adversely affected when cultured under the highest agar concentration of 8 g L⁻¹. There is a reverse analogous relationship between the gelling agent concentration and water potential - water and dissolved substances availability, thus the increase of the former results in the decrease of the latter (Smith and Spomer, 1995). The proliferation rate of *A. occulta* microshoots under study was substantially raised under the intermediate agar concentration of 7 g L⁻¹, however the further increase in its concentration to 8 g L⁻¹ had a negative effect. Shoot proliferation rate of plantlets cultured *in vitro* is reported to be declined with the increase in agar concentration (Yadav *et al.*, 2003) and the reduced regeneration capacity of explants could be ascribed to the lowering of water potential in the medium, derived from the increase in the osmotic potential imposed by higher gelling agent concentrations than the optimum (Smith and Spomer, 1995).

Different cytokinin types

In this study, BA at 4.4 µM gave better multiplication results as evidenced by the higher shoot number and proliferation rate than 4.4 µM KIN when combined with 0.44 µM IBA. Similar observations that are in line with our findings have been found by Marcu *et al.* (2014), who reported that BA was more favorable for shoot proliferation of *in vitro* cultures than KIN, and such response could be explained according to Buah *et al.* (2010) by the higher stability and thus longer persistence of BA in the medium. However, different cytokinin types, other than BA, were shown to be more beneficial for shoot proliferation attributes of *in vitro* cultures in other *Achillea* species including 5.6 µM KIN in *A. fragrantissima* (Younes *et al.*, 2015) and 3.4 µM TDZ in *A. millefolium* L. (Alvarenga *et al.*, 2015). The optimum concentration of PGRs for inducing shoot proliferation is species-dependent since KIN concentration was demonstrated to be higher than BA for exhibiting the same optimum result regarding multiple shoot formation and elongation in *Portulaca grandiflora* (Srivastava and Joshi, 2009). All four different cytokinin types studied herein had a positive effect as exhibited higher shoot numbers and proliferation rates in comparison to the control (cytokinins-free) with BA to be the most effective, whereas non-significant were the changes related to shoot length. The best conditions for *in vitro* shoot proliferation in other *Achillea* species were 4.4 µM BA + 2.8 µM IAA for *A. filipendulina* (Evenor and Reuveni, 2004), and 4 µM BA for *A. millefolium*, thus BA proved to be useful for micropropagation in comparison to either ZT or KIN (Shatnawi, 2013). In the studied *A. occulta*, shoot number was better stimulated by BA (2.55) compared with KIN (1.85), the length of produced shoots was higher in the presence of ZT (0.70 cm) than KIN (0.59 cm), proliferation rate was the highest with BA (1.66), while non-significant changes were noticed in shoot formation percentages (60-85%) irrespective of cytokinin type (BA, 2-ip, ZT, KIN). Based on the results obtained by Turker *et al.* (2009) in *A. millefolium* L., shoot-tip explants when cultured on MSMO medium enriched with either 13.32 µM BA + 2.85 µM IAA or 23.23 µM KIN + 4.92 µM IBA gave the highest shoot numbers at a 100% percentage. Zebarjadi *et al.* (2011) pointed out that the percentage of multiple shoot formation may be influenced only when the endogenous cytokinin level in the plant is low, therefore if this condition is not valid and its content inside the plant is high then the exogenous application of this plant growth regulator in the culture medium may have negative impact on proliferation. The differential response of the explants to different cytokinin types may be explained by differences occurring in their uptake, recognition by the cells, and/or the mechanism of the cytokinins action (Kim *et al.*, 2001). BA was the most effective cytokinin type for proliferation of *A. occulta* under study, followed by ZT, while KIN was the least effective. The superiority of BA, over the other cytokinins, might be attributed to its ability to increase the polyribosome content in the cells due to the production of D-type cyclin (CycD3), which stimulates the cell cycle progression (Pasternak *et al.*, 2000).

ZT application method and BA concentration

Filtration through microporous filters (0.22-0.45) is used for thermo-labile organic constituents such as vitamins, growth regulators and amino acids (Torres, 1989). Physical stability of adenine-based cytokinins (BA, KIN, 2-ip, ZT, TDZ) as affected by high temperatures and pressures, such as those within an autoclave cycle is of interest (Hart *et al.*, 2016). Even though the stability of KIN (Miller *et al.*, 1955) and zeatin riboside (Miller, 1974) during autoclaving has been studied, the majority of cited literature is anecdotal whether these cytokinins are heat-labile i.e. ZT and 2-ip (Iliev *et al.*, 2010) or not heat-labile i.e. BA and KIN (Panaia *et al.*, 2011). In the present study, shoot formation percentages and shoot numbers per explant were not affected from either ZT sterilization method (autoclaved, filtered) or cytokinins application form (BA, TZ, BA + TDZ). In consistency with our findings, non-significant was the difference in the results obtained in a soybean-bioassay in the case of sterilization method of zeatin riboside, exogenously applied either through autoclaving or filtration, without however measuring quantitatively or specifically its degradation (Miller, 1974).

In the studied *A. occulta*, on the one hand, shoot length was better stimulated when ZT was autoclave-sterilized and applied as a single cytokinin type, while filter-sterilized ZT without undergo autoclaving (1.1 bar, 121 °C for 20 min) adversely affected this parameter. In the same study, on the other hand, different response on shoot length was observed in the combined effect of both cytokinin types, BA + ZT, regardless of ZT sterilization method being non-statistically significant. According to Hart *et al.* (2016), the stability of ZT in MS medium-basal salts after one autoclave cycle of 1.1 bar 121 °C for 30 min revealed negligible amounts of degradation when dissolved at 10 mg L⁻¹ KOH. In our study, ZT before its incorporation into the culture medium, either autoclave-sterilized or filter-sterilized, it was dissolved in a relatively high base concentration (1.0 N KOH). The concentration of solvent in the case of KOH base for dissolving ZT prior its sterilization plays a significant role in the rate of its degradation loss, thus 20% of its mass can be lost over 18 days after being dissolved in 0.5 N KOH (Hart *et al.*, 2016).

Although ZT sterilization method hardly affected proliferation rate of the studied *A. occulta*, autoclave-sterilized ZT vs filter-sterilized ZT, and autoclave-sterilized ZT + BA vs filter-sterilized ZT + BA, the factor that played the key role was the presence of a single cytokinin (BA or ZT) or combined cytokinins (BA + ZT), regardless of ZT application method. It becomes clear that the stability of adenine-based cytokinins such as ZT in a range of aqueous solution conditions and through one autoclave cycle in a moderately alkaline solution (0.05 N KOH) is depended on several factors including crystallization and acidic conditions during extreme heating, which can lead to potential adenine degradation, therefore these compounds should be used with caution and administered properly for plant tissue culture (Hart *et al.*, 2016). In several woody species and in accordance with our findings, the combined use of ZT with BA proved to be more efficacious for shoot proliferation than ZT alone (Grigoriadou *et al.*, 2002; Ali *et al.*, 2009).

The effectiveness of various combinations of cytokinins on micropropagation of woody plants has been reported by Higuchi *et al.* (2004), however, shoot elongation phase is highly sensitive to higher concentrations of growth regulators (Kadota and Niimi, 2003). In this study, autoclave-sterilized ZT enhanced better the elongation of produced *A. occulta* microshoots, while BA applied either alone or with ZT (autoclave- or filter-sterilized) had a negative effect. A possible explanation for this outcome is that the excessive supply of both cytokinins (ZT + BA) has concealed the balance of cytokinins resulting in ethylene production which in turn inferred the inhibition of internode elongation due to cessation of cell division and expansion, suggesting interdependence of hormonal regulatory pathways (Higuchi *et al.*, 2004). Our results are not in line with those reported by Rugini *et al.* (1999) and Ali *et al.* (2009), as shoot elongation response to the combined use of ZT + BA was more effective than that of ZT alone, showing effect species-dependent.

In vitro rooting under different auxin types and concentrations

The exogenous application of auxins is essential especially in the early stages of rhizogenesis (Radmann *et al.*, 2014) and the emergence of numerous roots (Dobranszki and Silva, 2010) including IBA and NAA as synthetic auxin types, and indole-3-acetic acid (IAA) as the main auxin type occurring naturally within plants (Phillips and Garda, 2019). Regarding the studied *A. occulta*, IBA was the ideal auxin type and 2 μM its optimum concentration for maximum response related to rooting percentage (20%). Our findings are in line with the outcomes derived from a previous study conducted in the same plant species (*A. occulta*), wherein rooting of microshoots proved to be very difficult exhibiting a 12.5% rooting percentage after supplementing the MS medium with 20 μM IBA (Grigoriadou *et al.*, 2011). There are several reports in other *Achillea* species pointing out that the highest rooting percentages were achieved with 9.84 μM IBA in *A. biebersteinii* (Kahrizi and Kakaei, 2010), IAA (2.85 or 5.72 μM) or 2.69 μM NAA in *A. filipendulina* (Evenor and Reuveni, 2004), 9.84 μM IBA (Turker *et al.*, 2009; Shatnawi, 2013) and 3.23-10.74 μM NAA in *A. millefolium* (Shatnawi, 2013), and 5.72 μM IAA in *A. pyrenaica* Sibth. ex Godr (Marcu *et al.*, 2014). The better effectiveness of IBA compared with NAA on rooting induction of *A. occulta* in this study could be attributed to several reasons including (1) more resistance to photodegradation, adherence to microshoots, and inactivation by biological action (Elmongi *et al.*, 2018), (2) successive rooting gene activation, better uptake, transportation and stability (Liu *et al.*, 2017), (3) slow migration and delayed degradation (Mansseri-Lamrioui *et al.*, 2011), and (4) slower oxidization rate by the oxidase (Wolella, 2017) and thus of higher stability (Abbasi *et al.*, 2019). In this study, the highest concentration (5 μM) of either IBA or NAA, and the simultaneous application of both auxins (IBA + NAA) irrespective their concentration combination ratios were detrimental and toxic resulting in complete rooting inhibition, which might be ascribed to ethylene synthesis induced due to high auxin concentrations (Aghaye and Yadollahi, 2012).

Root elongation of *A. occulta* microshoots under study was best stimulated with 2 μM IBA. It has been formulated that low auxin concentrations are promotive on root growth and inhibitory at high ones (Baker and Wetzstein, 2004). Similarly to our results, 9.84 IBA had the best effect on root length in *A. biebersteinii*, whereas higher concentrations had a negative effect (Kahrizi and Kakaei, 2010). Different auxin types, other than IBA, were found to be more effective on root elongation in other *Achillea* species including IAA in *A. filipendulina* followed by IBA as compared with NAA (Evenor and Reuveni, 2004) and NAA in *A. millefolium* (Shatnawi, 2013). The increase of NAA concentration was accompanied by a successive decrease of root length in this study and this might be due to differences in the catabolism of this auxin (Evenor and Reuveni, 2004) as a result of differential transport of the various auxins (Yamamoto and Yamamoto, 1998).

In the current study, NAA was the ideal auxin type and 2 μM its optimum concentration for maximum response related to root number as compared to IBA regardless concentration. Our results come in contradiction with the results reported in other *Achillea* species including *A. filipendulina* where all three auxin types used (IBA, NAA, IAA) gave similar root numbers (Evenor and Reuveni, 2004), *A. millefolium* where root number was maximum with 6.85 μM IBA (Shatnawi, 2013), and *A. pyrenaica* where 5.7 μM IAA gave more roots compared with 5.4 μM NAA (Marcu *et al.*, 2014).

Ex vitro acclimatization, hardening and survival of rooted microshoots

In the present study, moderate non-significant survival rates, 50-66.67% were obtained for *A. occulta* rooted microshoots derived *in vitro* from media enriched with 2 μM IBA (66.67%) or 1 μM NAA (50%). It is quite common for *Achillea* species to show a wide range of *ex vitro* survival rates between 33% and 100% as has already been observed in *A. occulta* (33%) (Grigoriadou *et al.*, 2011), *A. millefolium* (70%) (Shatnawi, 2013), *A. filipendulina* (90%) (Evenor and Reuveni, 2004), *A. thracica* Velen. (90%) (Rogova *et al.*, 2015), and *A. fragrantissima* (100%) (Younes *et al.*, 2015) under greenhouse acclimatization conditions in a peat moss: perlite (1:1 v/v) mixture.

Conclusions

In this study, a successful reproducible micropropagation protocol of *A. occulta* is presented enabling its large-scale multiplication, domestication and germplasm conservation. The direct organogenesis protocol was established based on several culture media compositions and parameters for optimization, including different types and concentrations of PGRs as well as their ratio, different agar concentrations, different cytokinin types, different sterilization methods of ZT prior its addition to the culture medium, as well as different auxin types and concentrations. The optimized micropropagation protocol established in this study could be used as a first step tool in the future for the large-scale production of certain secondary metabolites from *in vitro* grown *A. occulta* plants contributing to further analyses related to their pharmacological, physiological and biochemical background with potential use in dermatology and cosmetic industry.

Authors' Contributions

Conceptualization: VS and KG; Data curation: VS; Formal analysis: VS; Funding acquisition: EM and KG; Investigation: VS; Methodology: VS and KG; Project administration: KG; Resources: EM and KG; Software: VS; Supervision: KG; Validation: KG; Visualization: VS; Writing - original draft: VS; Writing - review and editing: VS and KG. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

- Abbasi F, Khadivi A, Taghizadeh M, Valizadehkaji B (2019). Micropropagation of *Prunus scoparia*, a suitable rootstock for almond under drought conditions. International Journal of Fruit Science 19:221-230. <https://doi.org/10.1080/15538362.2018.1539695>
- Aghaye RNM, Yadollahi A (2012). Micropropagation of GF677 rootstock. Journal of Agricultural Science 4:131-138. <http://dx.doi.org/10.5539/jas.v4n5p131>
- Ali A, Ahmad T, Abbasi NA, Hafiz IA (2009). Effect of different media and growth regulators on *in vitro* shoot proliferation of olive cultivar 'Moraiolo'. Pakistan Journal of Botany 41:783-795.

- Alvarenga ICA, Silva ST, Bertolucci SKV, Pinto JEBP, Pacheco FV (2015) Application of thidiazuron (TDZ) for *in vitro* multiplication of yarrow (*Achillea millefolium* L.) and profile of volatile compounds. Australian Journal of Crop Science 9:948-953.
- Baker CM, Wetzstein HY (2004). Influence of auxin type and concentration on peanut somatic embryogenesis. Plant Cell, Tissue and Organ Culture 36:361-368. <https://doi.org/10.1007/BF00046094>
- Biswas A, Bari MA, Roy M, Bhadra SK (2011). *In vitro* propagation of *Stemona tuberosa* Lour. - A rare medicinal plant through high frequency shoot multiplication using nodal explants. Plant Tissue Culture & Biotechnology 21:151-159. <https://doi.org/10.3329/ptcb.v21i2.10238>
- Buah JN, Danso E, Taah KJ, Abole EA, Bediako EA, Asiedu J, Baidoo R (2010). The effects of different concentrations cytokinins on the *in vitro* multiplication of plantain (*Musa* sp). Biotechnology 9:343-347. <https://doi.org/10.3923/biotech.2010.343.347>
- Cellarova E, Grelakova K, Repcak M, Honcariv R (1982). Morphogenesis in callus tissue cultures of some *Matricaria* and *Achillea* species. Biologia Plantarum 24:430-433. <https://bp.ueb.cas.cz/pdfs/bpl/1982/06/08.pdf>
- Chaturvedi HC, Jain M, Kidwai NR (2007). Cloning of medicinal plants through tissue culture - A review. Indian Journal of Experimental Biology 45:937-948.
- Constantinidis T, Kalpoutzakis E (2009). *Achillea occulta* Constantin. & Kalpoutz. Vulnerable (VU). In: Phitos D, Constantinidis T, Kamari G (Eds). The red data book of rare and threatened plants of Greece. Vol 1 (A–D). Hellenic Botanical Society, Patras pp 40-42.
- Dimopoulos P, Raus T, Bergmeier E, Constantinidis Th, Iatrou G, Kokkini S, Strid A, Tzanoudakis D (2016). Vascular plants of Greece - An annotated checklist. Supplement. Willdenowia 46:301-347. <https://doi.org/10.3372/wi.46.46303>
- Dobranski J, Silva JAT (2010). Micropropagation of apple - A review. Biotechnology Advances 28:462-488. <https://doi.org/10.1016/j.biotechadv.2010.02.008>
- Elmogy MS, Cao Y, Zhou H, Xia Y (2018). Root development enhanced by using indole-3-butyric acid and naphthalene acetic acid and associated biochemical changes of *in vitro* azalea microshoots. Journal of Plant Growth Regulation 37:813-825. <https://doi.org/10.1007/s00344-017-9776-5>
- Evenor D, Reuveni M (2004). Micropropagation of *Achillea filipendulina* cv. 'Parker'. Plant Cell, Tissue and Organ Culture 79:91-93. <https://doi.org/10.1023/B:TICU.0000049441.48404.70>
- Figueiredo ACS, Pais MSS (1991). *Achillea millefolium* (yarrow) cell suspension cultures: Establishment and growth conditions. Biotechnology Letters 13:63-68. <https://doi.org/10.1007/BF01033519>
- Ghasempour H, Dabiri S, Cheraghi M (2012). Composition of the volatile oil, tissue culture and micro-regeneration optimization of wild yarrow (*Achillea biebersteinii*). Iranian Journal of Plant Physiology 2:539-546.
- Grigoriadou K, Krigas N, Maloupa E (2011). GIS-facilitated *in vitro* propagation and *ex situ* conservation of *Achillea occulta*. Plant Cell, Tissue and Organ Culture 107:531-540. <https://doi.org/10.1007/s11240-011-0004-x>
- Grigoriadou K, Vasilakakis M, Eleftheriou EP (2002). *In vitro* propagation of the Greek olive cultivar 'Chondrolia Chalkidikis'. Plant Cell, Tissue and Organ Culture 71:47-54. <https://doi.org/10.1023/A:1016578614454>
- Hart DH, Keightley A, Sappington D, Nguyen PTM, Chritton C, Seckinger GR, Torres KC (2016). Stability of adenine-based cytokinins in aqueous solution. *In Vitro Cellular and Developmental Biology - Plant* 52:1-9. <https://doi.org/10.1007/s11627-015-9734-5>
- Higuchi M, Pischke MS, Mahonen AP (2004). *In planta* functions of the *Arabidopsis* cytokinin receptor family. Scientia Horticulturae 45:366-388. <https://doi.org/10.1073/pnas.0402887101>
- Holobiuc I, Catană R, Cogălniceanu G, Cristea V (2018). Biotechnological approach for *ex-situ* conservation of the vulnerable species *Moehringia jankae*. Romanian Biotechnological Letters 23:954-963.
- Iliev I, Gajdošová A, Liviaková G, Jain SM (2010). Plant micropropagation. In: Davey MR, Anthony P (Eds). Plant cell culture: Essential methods. Wiley-Blackwell, West Sussex, UK pp 1-24.
- IUCN (2001). IUCN red list categories and criteria. Version 3.1. IUCN Species Survival Commission. Gland and Cambridge. Retrieved 2023 March 16 from http://www.iucnredlist.org/documents/redlist_cats_crit_en.pdf
- Kadota M, Niimi Y (2003). Effects of cytokinin types and their concentrations on shoot proliferation and hyperhydricity on *in vitro* pear cultivar shoots. Plant Cell, Tissue and Organ Culture 72:261-265. <https://doi.org/10.1023/A:1022378511659>

- Kahrizi D, Kakaei M (2010). Effect of 6-benzylaminopurine, 2,4-dichlorophenoxyacetic acid and indole-3-butyric acid on micropropagation stages of *Achillea biebersteinii*. Asian Journal of Chemistry 22:2383-2386.
- Kim KH, Park HK, Park MS, Yea UD (2001). Effects of auxin and cytokinin on organogenesis of soybean *Glycine max* L. Journal of Plant Biotechnology 3:95-100.
- Liu J, Moore S, Chen C, Lindsey K (2017). Crosstalk complexities between auxin, cytokinin, and ethylene in *Arabidopsis* root development: from experiments to systems modeling, and back again. Molecular Plant 10:1480-1496. <https://doi.org/10.1016/j.molp.2017.11.002>
- Manole-Păunescu A (2014). Biotechnology for endangered plant conservation. In: Ahuja MR, Ramawat KG (Eds). Biotechnology and biodiversity. Springer, Switzerland pp 181-202. https://doi.org/10.1007/978-3-319-09381-9_10
- Mansseri-Lamrioui A, Louerguioui A, Bonaly J, Yakoub-Bougdal S, Allili N, Gana-Kebbouche S (2011). Proliferation and rooting of wild cherry: The influence of cytokinin and auxin types and their concentration. African Journal of Biotechnology 10:8613-8624. <https://doi.org/10.5897/AJB11.450>
- Marcu D, Halmágyi A, Besenyi E, Clapa D, Fira A, Cristea V (2014). *In vitro* conservation of *Achillea pyrenaica* Sibth. ex Godr., a Pyrenean endemic species. Muzeul Olteniei Craiova Oltenia Studii și comunicări Științele Naturii 30:55-60. http://biozoojournals.ro/oscsn/cont/30_2/08_Marcu_pp55-60.pdf
- Miller CO (1974). Ribosyl-*trans*-zeatin, a major cytokinin produced by crown gall tumor tissue. Proceedings of the National Academy of Sciences of the United States of America 71:334-338. <https://doi.org/10.1073/pnas.71.2.334>
- Miller CO, Skoog F, Von Saltza MHV, Strong FM (1955). Kinetin, a cell division factor from deoxyribonucleic acid. Journal of the American Chemical Society 77:1392. <https://doi.org/10.1021/ja01610a105>
- Mohammadhosseini M, Sarker SD, Akbarzadeh A (2017). Chemical composition of the essential oils and extracts of *Achillea* species and their biological activities: A review. Journal of Ethnopharmacology 199:257-315. <https://doi.org/10.1016/j.jep.2017.02.010>
- Monteiro ACBA, Higashi EN, Goncalves AN, Rodriguez PM (2000). A novel approach for the definition of the inorganic medium components for micropropagation of yellow passion fruit (*Passiflora edulis* Sims. f. *flavicarpa* Deg.). In Vitro Cellular and Developmental Biology - Plant 36:527-531. <https://doi.org/10.1007/s11627-000-0094-3>
- Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15:473-497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- Panaia M, Bunn E, McComb J (2011). Primary and repetitive secondary somatic embryogenesis of *Lepidosperma drummondii* (Cyperaceae) and *Bloskion tetraphyllum* (Restionaceae) for land restoration and horticulture. In Vitro Cellular and Developmental Biology - Plant 47:379-386. <https://doi.org/10.1007/s11627-010-9335-2>
- Pasternak T, Miskolczi P, Ayaydin F, Mészáros T, Dudits D, Feher A (2000). Exogenous auxin and cytokinin dependent activation of CDKs and cell division in leaf protoplast-derived cells of alfalfa. Plant Growth Regulation 32:129-141. <https://doi.org/10.1023/A:1010793226030>
- Păunescu A (2009). Biotechnology for endangered plant conservation: A critical overview. Romanian Biotechnological Letters 14:4095-4103. https://doi.org/10.1007/978-3-319-09381-9_10
- Phillips GC, Garda M (2019). Plant tissue culture media and practices: An overview. In Vitro Cellular and Developmental Biology - Plant 55:242-257. <https://doi.org/10.1007/s11627-019-09983-5>
- Radmann EB, Gallo CM, Ritterbusch CW, Bianchi VJ, Fernando JA, Peters JA (2014). *In vitro* rooting and acclimatization of 'Mr. S. 2/5' plum rootstock. Plant Cell & Micropropagation 10:21-31.
- Rogova M, Ganeva T, Stefanova M, Koleva D, Toteva VK (2015). Morphoanatomical study of *in vitro* propagated and *ex vitro* adapted *Achillea thracica* Velen. plants. Bulgarian Journal of Agricultural Science 21:947-950.
- Rugini E, Gutierrez-Pesce P, Sampinato PL (1999). New perspective for biotechnologies in olive breeding: Morphogenesis, *in vitro* selection and gene transformation. Acta Horticulturae 474:107-110. <https://doi.org/10.17660/ActaHortic.1999.474.18>
- Shatnawi MA (2013). Multiplication and cryopreservation of yarrow (*Achillea millefolium* L., Asteraceae). Journal of Agriculture, Science and Technology 15:163-173.
- Smith MAL, Spomer LA (1995). Vessels, gels, liquid media, and support systems. In: Aitken-Christie J, Kozai T, Smith ML (Eds). Automation and environmental control in plant tissue culture. Springer, Dordrecht pp 371-404. https://doi.org/10.1007/978-94-015-8461-6_16

- Srivastava A, Joshi AG (2009). *In vitro* behavior of nodal explants of *Portulaca grandiflora* under the influence of cytokinins. *Acta Universitatis Latviensis Biology* 753:43-48.
- Stojanovic G, Radulovic N, Hashimoto T, Palic R (2005). *In vitro* antimicrobial activity of extracts of four *Achillea* species: The composition of *Achillea clavennae* L. (Asteraceae) extract. *Journal of Ethnopharmacology* 101:185-190. <https://doi.org/10.1016/j.jep.2005.04.026>
- Strzpek-Gomólka M, Gawel-Bęben K, Kukula-Koch W (2021). *Achillea* species as sources of active phytochemicals for dermatological and cosmetic applications. *Hindawi Oxidative Medicine and Cellular Longevity* 2021:6643827. <https://doi.org/10.1155/2021/6643827>
- Torres KC (1989). Tissue culture techniques for horticultural crops. Springer, New York. <https://doi.org/10.1007/978-1-4615-9756-8>
- Turker AU, Yucesan B, Gurel E (2009). *In vitro* regeneration of *Achillea millefolium* L. from shoot-tips and root segments of seedlings. *Journal of Plant Biochemistry and Biotechnology* 18:65-69. <https://doi.org/10.1007/BF03263297>
- Wawrosch C, Kopp B, Kubelka W (1994). *In vitro* propagation of *Achillea asplenifolia* Vent. through multiple shoot regeneration. *Plant Cell Reports* 14:161-164. <https://doi.org/10.1007/BF00233782>
- Wolella EK (2017). Surface sterilization and *in vitro* propagation of *Prunus domestica* L. cv. Stanley using axillary buds as explants. *Journal of Biotech Research* 8:18-26.
- Yadav MK, Gaur AK, Garg GK (2003). Development of suitable protocol to overcome hyperhydricity in carnation during micropropagation. *Plant Cell, Tissue and Organ Culture* 72:153-156. <https://doi.org/10.1023/A:1022236920605>
- Yamamoto M, Yamamoto KT (1998). Differential effects of 1-naphthaleneacetic acid, indole-3-acetic acid and 2,4-dichlorophenoxyacetic acid on the gravitropic response of roots in an auxin-resistant mutant of *Arabidopsis* aux1. *Plant Cell Physiology* 39:660-664. <https://doi.org/10.1093/oxfordjournals.pcp.a029419>
- Younes LS, Shibli RA, Al-Qudah TS (2015). *In vitro* propagation and acclimatization of *Achillea fragrantissima* Frossk Sch. Bip. *Jordan Journal of Agricultural Sciences* 11:339-351.
- Zebarjadi AR, Najafi SH, Ghasempour HR, Motamedi J (2011). Establishment of a practical tissue culture for producing hairy roots of *Valeriana officinalis* L. via *Agrobacterium rhizogenes*. *Journal of Medicinal Plants Research* 5:4984-4992.



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