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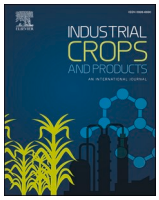

In vitro propagation of *Alkanna tinctoria* Tausch.: a medicinal plant of the Boraginaceae family with high pharmaceutical value

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ABSTRACT

Alkanna tinctoria Tausch. (Boraginaceae), commonly known as alkanet or dyers' bugloss/alkanet, is a perennial plant rich in naphthoquinone enantiomers, such as Alkannin and Shikonin (A/S), which possess a wide range of pharmaceutical properties and are used as cosmetics, food additives, and natural dyes. This plant is mostly exploited from the wild, increasing the risk of its extinction as reported for other A/S producing plants extracted from their natural environment. Its cultivation under controlled conditions remains difficult and the need for alternative production systems both for preserving this endangered species and for increasing the production of A/S at a marketable level, has become a necessity. In the present study, a protocol for the *in vitro* production of *A. tinctoria* plants using shoot-tip explants was developed. Several culture media, concentrations of hormones, sugar, and gelling agents were tested to improve proliferation, rooting, and acclimatization of micropropagated shoot-tip explants from plants collected in the wild. Surface disinfection was optimal after immersion of shoot-tips and/or nodal explants in Signum® fungicide (30 min), ethanol 70% (1 min), and sodium hypochlorite 3% (10 min). Shoot proliferation was the highest on Murashige-Skoog basal medium enriched with 1.1 μM 6-benzyladenine, 0.15 μM α-naphthalene acetic acid, and 0.3 μM gibberellic acid with a proliferation rate of 3.9 every two weeks. For rooting, the Root Culture 1 (RC1) modified medium free of ammonium nitrate and enriched with 2.85 μM indole-3-acetic acid was the more adequate with 80% of roots formation after 30 days. Finally, acclimatization was optimal (100% survival rate) following transfer of the rooted explants in pots containing a peat moss:perlite (1:1, v/v) mixture, kept under a 90% relative humidity fog system for 10 days, followed by a decrease in relative humidity of 5% every day until 40% and a gradual increase in light intensity. The protocol developed allowed the production under *in vitro* culture conditions of a sufficient number of *A. tinctoria* plants with high levels of *ex vitro* survival, opening the door to industrial exploitation of its secondary metabolites and to the conservation of this important medicinal plants.

1. Introduction

Alkanna tinctoria Tausch. (family Boraginaceae) is a perennial herbaceous plant with a central distribution in the Mediterranean region (Valdés, 2011). It has a long history of medicinal uses characterized by its major active compounds, the naphthoquinone enantiomers Alkannin and Shikonin (A/S) (Papageorgiou et al., 1999). These compounds and their derivatives possess a wide range of medicinal properties (*e.g.*, wound healing, antioxidant, antimicrobial, anti-inflammatory, and anti-cancer) and are used as well as cosmetics, food additives or dyes (Assimopoulou et al., 2004; Papageorgiou et al., 2008; Malik et al., 2016).

Several efforts have been made to produce A/S from plants grown under controlled conditions. Cell tissues and root cultures of *A. tinctoria* as well as cell suspensions of *Arnebia* spp. in stirred-tank and air-lift bioreactors have been attempted, but the level of production remained insufficient (Urbanek et al., 1996; Gerardi et al., 1998; Gupta et al., 2014). The only successful example of Shikonin scaling up from cell

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cultures was with *Lithospermum erythrorhizon* Sieb. et Zucc. (Yazaki, 2017). Yet, A/S derivatives remains mostly extracted from roots of plant grown in nature, such as *A. tinctoria,* resulting in an unconsidered exploitation and risk of extinction for this important medicinal plant.

The cultivation of Boraginaceae following conventional agriculture practices is not feasible due to poor seed germination, low-availability of planting material, extensive time needed for noticeable A/S derivatives production, and high harvesting costs (Malik et al., 2016). For these reasons, direct plant regeneration and micropropagation are options that have been considered in the recent years for several Boraginaceae, such as *Arnebia hispidissima* (Lehm). DC. (Pal and Chaudhury, 2010), *Sericostoma pauciflorum* Stocks ex Wight (Satish et al., 2014), and *Mertensia maritima* L. (Park et al., 2019). These methods are useful for the conservation and propagation of plants, and for fulfilling the demands of pharmaceutical industries. For instance, the total content of Shikonin from 1 g of induced *A. hispidissima* root tissue was 0.50 mg after 50 days of *in vitro* culture (Pal and Chaudhury, 2010).

The *in vitro* cultivation of *A. tinctoria* may represent a viable and innovative method to maintain and produce sufficient number of plants, further opening the doors to pharmaceutical exploitation. However, no improvement in the cultivation or either in the development of an exhaustive *in vitro* propagation protocol have been made so far. Therefore, the objective of this study is to report a successful protocol for the *in vitro* production of *A. tinctoria* plants using shoot-tip explants, and their full acclimatization to *ex vitro* conditions. The method and growth conditions are detailed and potential for mass-production discussed.

2. Materials and methods

2.1. Collection of plant material

One botanical expedition to collect wild-growing *A. tinctoria* plants was conducted on the 19 July 2017, in a suburban pine forest, altitude 50 m of Northern Greece (Evaggelistria, Seih Sou, Thessaloniki). A special collection permit, obtained by the Institute of Plant Breeding and Genetic Resources, Hellenic Agricultural Organization Demeter (IPBGR, HAO Demeter), which is issued annually by the Greek Ministry of Environment and Energy, was used. The habitat collection of the plant material was described as a rocky opening in a pine forest, temperate and sub-Mediterranean grassland, xeric Mediterranean Phrygana and grassland. The wild plants collected were taxonomically identified and were given the International Plant Exchange Network (IPEN) accession number GR-1-BBGK-17,5975 for their long-term *ex situ* conservation (including also sexual, asexual – vegetative, and *in vitro* propagation trials) at the premises of IPBGR, HAO-Demeter.

2.2. A. tinctoria ex situ conservation

A. tinctoria was propagated asexually by cuttings. Six rooting trials were conducted during summer – autumn 2017. In total, 451 softwood top cuttings (3–4 cm long) were treated by immersion of their base in a 2000 mg L^{-1} indole-3-butyric acid (IBA) solution for 10 s. The cuttings were then transferred in 76 mL pots (4.15×4.05×7 cm) containing a peat moss (Terrahum, Klasmann-Deilmann Gmbh, Germany) and perlite (Geoflor, Perlite Hellas S.A., Greece) (1:3, v/v) mixture. After 20 days of growth in the heated mist of the greenhouse [18–21 °C soil, 25–30 °C/18–25 °C day/night air temperature, and 65–80% relative humidity (RH)], plants' rooting was checked (Fig. 1a-b). Then, the plants were maintained, for further experimentation, in 0.33 L square plastic pots (7×7x8 cm) containing a peat moss (TS2 basic substrate medium, Klasmann-Deilmann Gmbh, Germany) and perlite (3:1, v/v) mixture under unheated-greenhouse conditions (Fig. 1c-d). Soil and air temperature, and RH were different due to seasonal variability of the IPBGR, HAO-Demeter greenhouse (*i.e.*, during summer: 18–22 °C soil, 25–35 °C air, and 60–70% RH; during autumn: 18–20 °C, 15–25 °C air, and 70–85% RH; during winter: 17–19 °C soil, 5–15 °C air, and 85–99% RH).

2.3. Disinfection and initial establishment in vitro of plant material

Nine disinfection protocols were compared, during autumn-winter 2017 and late spring-early summer 2018, for the establishment of *in vitro* contaminant-free plant material. Shoot-tips or shoot intermediate nodes (1–1.5 cm) were detached with scalpels from the mother plants above to proceed with the disinfection as follows: soaked in Signum® fungicide 26.7/6.7 WG (0.070 g /100 mL ddH_2O, BASF Agricultural solutions, The Netherlands) for 30 min, then bathed or not in 70% ethanol for 1 min and in sodium hypochlorite (NaOCl) solutions at different concentrations (1, 2, 3%) and duration of immersion (3, 10, 15 min) under continuous agitation. The explants were finally rinsed 4–5 times with sterilized deionized water and placed individually in borosilicate glass test tubes (100×25 mm) containing Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 20 g L^{-1} sucrose (Duchefa, The Netherlands) and 6 g L^{-1} Plant Agar (Duchefa, The Netherlands). The successfully established contaminant-free explants were sub-cultured every 3–4 weeks. Eight to 10 successive subcultures were done during 8 months until a sufficient number of plants was produced for further experimentation. The cultures were maintained in a growth chamber under 16 h/8 h light/dark photoperiod, temperature of 22 ± 1 °C (day/night), 80% RH, and cool white fluorescent light (PHILIPS, 36 W/830 G13 1214 mm) intensity of about 40 μmol $m^{-2}s^{-1}$. The percentage of contaminant-free and microbial-contaminated explants were recorded after 20 days of culture.

2.4. In vitro shoot proliferation

Three proliferation experiments were conducted using shoot-tip explants derived from the *in vitro* cultures above. The explants were transferred into Magenta™ vessels (Baby food jars, 62.4 ×95.8 mm, size: 200 mL, Sigma-Aldrich, Merck KGaA, Germany), cover by Magenta™ B-caps, and containing 35 mL of the culture media described below. The cultures were maintained in a growth chamber under the same conditions as described above. In the first experiment, three different culture media were tested, MS, Woody Plant Medium (WPM) (Lloyd and McCown, 1980), and Gamborg B5 (GB5) (Gamborg et al.,

Fig. 1. *Ex situ* conservation of *Alkanna tinctoria* plants: (a, b) asexual propagation by cuttings from wild-growing plants collected on mid-summer (July 2017); (c, d) vegetative growth and blooming of mother plants inside the greenhouse in following spring (March 2018).

1968), all enriched with 1.1 µM 6-benzyladenine (BA), 0.15 µM α-naphthalene acetic acid (NAA), and 0.3 µM gibberellic acid (GA_3) (Fig. 2a-b). Chemical composition of the basal culture media is presented in Supplementary Table S1. In the second experiment, two different gelling agents [6 g L^{-1} Plant Agar and 3 g L^{-1} Gelrite (Duchefa, The Netherlands)] in combination with MS and WPM culture media enriched with 1.1 µM BA, 0.15 µM NAA, and 0.3 µM GA_3 were tested (Fig. 2c-d). In the third experiment, MS culture medium PGR's-free supplemented with two sucrose and Plant Agar concentrations (20/30 g L^{-1} and 6 /7 g L^{-1}, respectively) was tested.

After 15 days of culture for the first and 20 days for the second and third proliferation experiment, the following measurements were recorded: shoot formation percentage, number of new shoots per explant, shoot length, proliferation rate (*i.e.*, the mean number of new non-hyperhydric shoots of at least 1 cm height per inoculated shoot-tip explant, expressed as the ratio between non-hyperhydric-proliferated and total number of initial explants), and hyperhydricity percentage. In the first and second experiment, 20 explants distributed in 4 vessels (each containing 5 explants) were considered per treatment, while in the third experiment, 18 explants distributed in 3 vessels (each containing 6 explants) were considered per treatment.

2.5. In vitro rooting

Three rooting experiments were conducted using shoot-tip explants derived from the *in vitro* shoot proliferation above. The explants were transferred into Magenta™ vessels and maintained in a growth chamber under the same conditions as described in Section 2.3. In the first experiment, full-strength MS and ½ MS in macro and micro-nutrients and FeNaEDTA were tested in combination with three different IBA concentrations (0, 2.5, 5 µM). In the second experiment, MS basal medium was enriched with two different NAA concentrations (2.69 and 5.4 µM) and combined with three different IBA concentrations (0, 2.5, 5 µM). In the third experiment, MS and RC1 NH_4NO_3-free culture media (Shimomura et al., 1991), both supplemented with indole-3-acetic acid (IAA) at four concentrations (0, 2.85, 5.71, 11.42 µM), were tested (Fig. 3a-d). Chemical composition of RC1 and ½ MS is presented in Supplementary Table S1.

After 20 days of culture for the first and second, and 30 days for the third rooting experiment, the following parameters were recorded: root numbers per rooted explant, root length, rooting and hyperhydricity percentages. In the first and third experiment, 20 explants distributed in 4 vessels (each containing 5 explants) were considered per treatment, while in the second experiment, 15 explants distributed in 3 vessels (each containing 5 explants) were considered per treatment.

2.6. Ex vitro acclimatization

Ex vitro acclimatization was conducted using rooted explants from the *in vitro* rooting experiments above, after being rinsed with tap water to remove the adhering medium (Fig. 3d-h). The rooted explants (10 replicates per culture media) were subsequently planted in 76 mL pots (4.15 ×4.05 ×7 cm) filled with a peat moss (Terrahum):perlite (1:1, v/v) mixture. The pots were placed in greenhouse under a 90% RH fog system, 25–30 °C/18–25 °C day/night air temperature, and 50% shading for 10 days. For the following 10 days, RH was reduced by 5% every day until 40%, while the light intensity was gradually increased to 250 µmol $m^{-2}s^{-1}$. The number of successfully acclimatized explants was recorded 40 days after transplanting in the greenhouse and was expressed as survival percentage. The plants were then transferred into 0.33 L pots for 20 days and then into 2.5 L pots containing a white peat moss (TS2, Klasmann):perlite (3:1, v/v) mixture. The pots were transferred to the greenhouse (summer conditions: 25–30 °C/18–25 °C day/night air temperature, approximately 16 h light duration of about 250 µmol $m^{-2}s^{-1}$ intensity, and 45–55% RH) and the plants were watered by sprinkling. After 60 days, the plants were transferred outside the greenhouse nursery to the external environment (28–35 °C/20–28 °C day/night air temperature and 50–55% RH, under a 50% shading net), where their *ex vitro* acclimatization was completed.

2.7. Statistical analysis

For all the experiments presented, vessels were randomized and plant growth parameters analyzed by one-way ANOVA followed by Duncan's multiple range test ($P \leq 0.05$) in order to discriminate among the means between the treatments. For all parameters, normal distribution of residuals variance and normality were checked before analyses. If the assumptions were not verified, the non-parametric Kruskal-

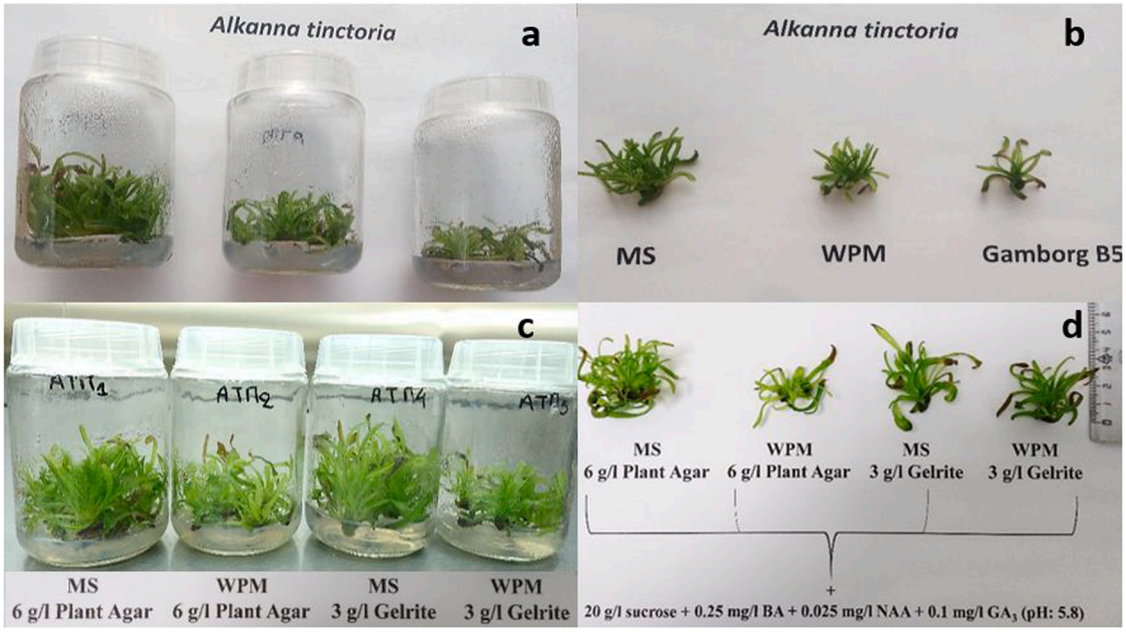

Fig. 2. *In vitro* proliferation of *Alkanna tinctoria* explants: (a, b) MS, WPM, and GB5 culture media enriched with 1.1 µM BA, 0.15 µM NAA, 0.3 µM GA_3; (c, d) MS and WPM culture media solidified with different gelling agents (Plant Agar and Gelrite), and enriched with 1.1 µM BA, 0.15 µM NAA, 0.3 µM GA_3.

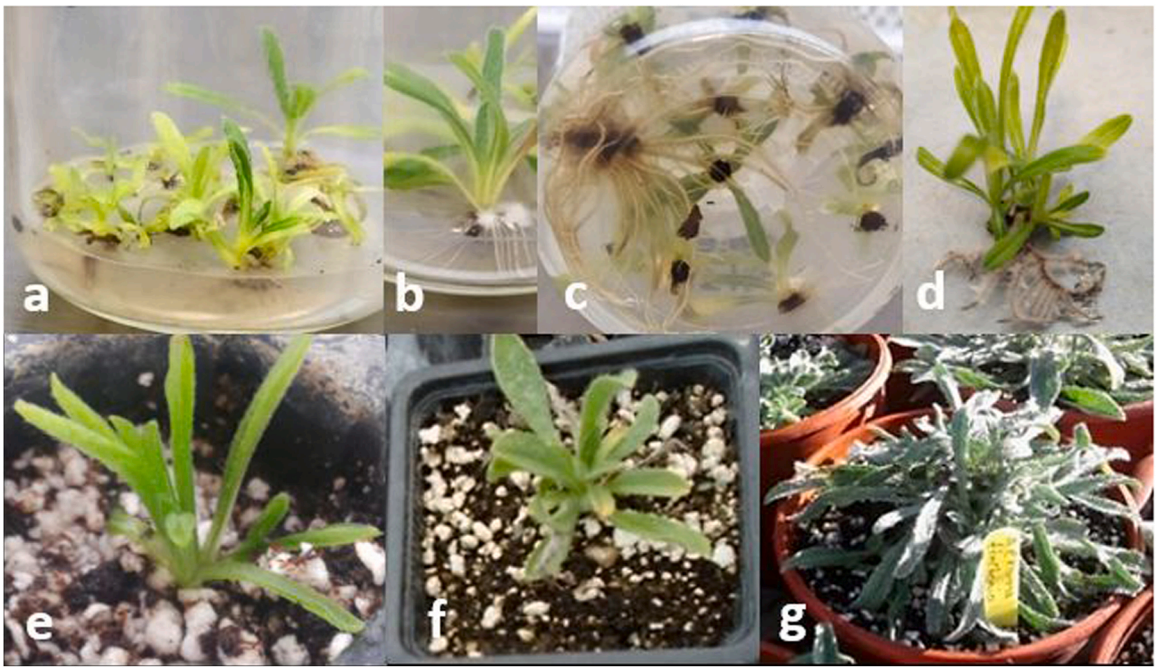

Fig. 3. *In vitro* rooting and *ex vitro* acclimatization of *Alkanna tinctoria* explants: (a-c) rooted explants in RC1 with 2.85 μM IAA medium; (d-f) vegetative growth of acclimatized plants after 5 and 40 days, respectively, in trays in the fog system; (g) *A. tinctoria* transplanted in 0.33 L pots inside the greenhouse.

Wallis test (K independent samples) was adopted. Data analyses were performed by SPSS Statistics for Windows, version 17 (SPSS Inc., Illinois, New York, USA).

3. Results and discussion

Different types of explants and tissue culture techniques have been used for growing and maintaining Boraginaceae species *in vitro*: callus cultures for *A. tinctoria* (Urbanek et al., 1996) and *Onosma bulbotrichom* DC. (Bagheri et al., 2018), root and suspension cultures for *L. erythrorhizon* (Tatsumi et al., 2016), suspension cultures for *Echium italicum* L. (Zare et al., 2010), callus and suspension cultures for *A. hispidissima* (Singh and Sharma, 2014). Although plant cell cultures have shown great potential to produce an array of valuable products, limited success has been achieved at the industrial scale. Yet, as mentioned before, A/S derivatives remains mostly extracted from roots of plant grown in nature, leading to the over-exploitation of important medicinal plants (Papageorgiou et al., 2008). Based on the literature available, *A. tinctoria in vitro* plants production using shoot-tip explants has never been attempted so far, and may thus represent a promising and potentially easy approach for large-scale production of *in vitro*/*ex vitro* plants. In the present study, a protocol for the *in vitro* production and *ex*

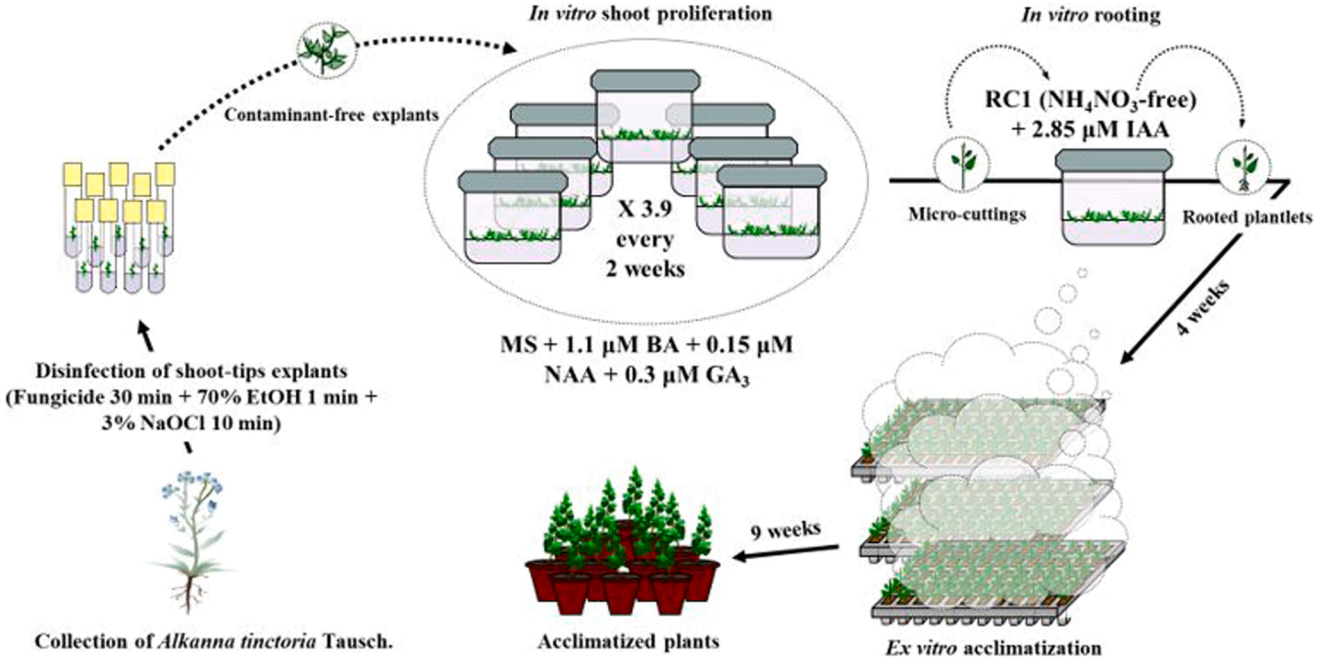

Fig. 4. Outline schema of the *in vitro* cultivation and *ex vitro* acclimatization protocol of *Alkanna tinctoria* explants.

vitro acclimatization of *A. tinctoria* was developed, aimed at solving the problem of plants availability, and possibly helping in the development of innovative approaches for the production of A/S, which are important pharmacological compounds.

The more suitable protocol can be summarized as follows (Fig. 4): (1) *disinfection* of shoot-tip explants by a combination of Signum® fungicide (30 min), ethanol 70% (1 min), and NaOCl 3% (10 min). (2) *Proliferation* of the surface-disinfected explants on MS medium enriched with 1.1 μM BA, 0.15 μM NAA, and 0.3 μM GA_3, supplemented with 20 g L^{-1} sucrose and solidified with 6 g L^{-1} Plant Agar. The explants are sub-cultured every 2 weeks, with a proliferation rate of about 3.9. (3) *Rooting* of the explants for a period of 4 weeks on RC1 medium (NH_4NO_3-free) supplemented with 2.85 μM IAA. (4) *Transfer* of the rooted explants in pots filled with a peat moss:perlite (1:1, v/v) mixture, kept under a 90% RH fog system for 10 days, followed by a decrease in RH of 5% every day until 40% and a gradual increase in light intensity. After 9 weeks, the explants are transferred into 2.5 L pots containing a white peat moss:perlite (3:1, v/v) mixture. Starting from 20 *A. tinctoria* contaminant-free explants sub-cultured 2 times in MS medium (4 weeks), rooted in RC1 (4 weeks) and acclimatized *ex vitro* (9 weeks), around 244 plants can be obtained fully adapted to the external environment.

The disinfection protocol reported above yielded the best percentage of successfully disinfected starting explant material close to 50% (Table 1). Regarding the *in vitro* shoot proliferation, the best result was obtained in MS medium (Table 2.I). Indeed, shoot formation was noticed in 100% of the explants with a proliferation rate of 3.9. This could be related to the higher macronutrients content, especially nitrogen (KNO_3 and NH_4NO_3), as compared with the two other culture media tested, WPM and GB5 (Arab et al., 2014). Moreover, the result obtained is consistent with the study of Yaman et al. (2019) on *Alkanna orientalis* (L.) Boiss. and *Alkanna sieheana* Rech. Fil., which reported a high percentage of shoot formation (30–66.6%), number, and length of shoots on MS medium containing 5.72 μM IAA. Improved shoot proliferation using different explant types (*e.g.*, nodal segments, callus, leaf lamina, petioles) proliferated in MS medium supplemented with different PGRs (*e.g.*, 1 N6-benzylaminopurine – BAP, IBA, kinetin, NAA) concentrations was also reported for other Boraginaceae plant species: *Rotula aquatica* Lour. (Martin, 2003), *Cordia verbenacea* DC. (Lameira and Pinto, 2006), *A. hispidissima* (Phulwaria and Shekhawat, 2013), *Echium orientale* L. (Turker et al., 2018). Several studies have described that the combination of cytokinins and auxins improve shoot proliferation in Boraginaceae plants (Pal and Chaudhury, 2010; Mahesh and Jeyachandran, 2013). Although 6-benzyladenine (BA) has often been considered as the prior cytokinin type used, because it is readily metabolized in plant tissues and can lead to the production of natural hormones (zeatin) (Rai et al., 2009), its use can lead to hyperhydricity symptoms (Ivanova and Van Staden, 2011) a phenomenon noticed as well in the present study. In order to improve the quality of the explants, two different gelling agents (Plant Agar and Gelrite) in combination with MS and WPM media were tested (Table 2.II). The percentage of shoot formation and number of new shoots per explant was significantly lower in WPM + Plant Agar treatment, while shoot length was significantly higher in MS+ Plant Agar medium. Moreover, the highest proliferation rate was confirmed in MS medium, characterized as well by a lower, but not significant, hyperhydricity percentage as compared with WPM medium and Gelrite gelling agent applied. Indeed, the symptoms remained quite evident in all the treatments tested at this stage. The slightly lower intensity of hyperhydric *A. tinctoria* explants on medium solidified with Plant Agar could be explained by the presence of a sulphated galactan (Nairn et al., 1995), which is involved in the control of hyperhydricity (Ivanova and Van Staden, 2011). Moreover, Gelrite has been reported to increase hyperhydricity symptoms in various plant species due to its physical structure, which allows a better absorption of cytokinins, NH_4^+, and water, suspected to be responsible of hyperhydricity (Ivanova and Van Staden, 2011). To investigate further and obtain a higher shoot proliferation rate, different sucrose and Plant Agar concentrations (20/30 g L^{-1} and 6/7 g L^{-1}, respectively) were tested (Supplementary Table S2). However, both parameters did not have a significant impact on the proliferation rate and hyperhydric shoots' percentage. Regarding the sucrose concentration, this result contradicts the study conducted on another Boraginaceae plant, *Rindera umbellata* (Waldst. & Kit.) Bunge, which reported that the highest percentage of explants with developed buds was achieved with a sucrose concentration of 0.06 M (± 20 g L^{-1}) as compared with 0.1 M (± 30 g L^{-1}) (Perić et al., 2012). Sucrose concentrations, higher than the optimum for shoot proliferation, could delay the development of cultured cells, leading to a decrease in the nutrient's uptake (Wu et al., 2006), lowering the water potential of the medium (Shim et al., 2003), and inducing osmotic stress (Shohael et al., 2006). In the present study, higher sucrose concentration (30 g L^{-1} instead of 20 g L^{-1}) did not inhibit explants proliferation, indicating that both sucrose concentrations were not supra-optimal and that they were perceived by cells as chemical signals, but without acting as stress agents (Teixeira da Silva, 2004).

For several Boraginaceae, the MS medium full or half-strength, proved to be effective for the *in vitro* rooting once enriched with different PGRs, such as IBA for *M. maritima* (Park et al., 2017), NAA for *R. aquatica* (Martin, 2003), and IBA + NAA + kinetin for *Arnebia pulchra* (Willd. ex Roem. & Schult.) Edm. (Ezati et al., 2015). In the current study, neither the individual application of IBA, NAA, and IAA nor the combination of IBA + NAA in MS medium gave satisfactory rooting results for *A. tinctoria* explants. Indeed, no roots were produced in MS + IAA treatments. The highest rooting (50%) and root number (3.2 roots per rooted explant) was obtained in MS + 5 μM IBA (Table 3.I), while higher root length (1.2 cm) was evident in MS + 2.69 μM NAA + 5 μM IBA (Table 3.II). Indole-3-butyric acid is characterized by higher stability than IAA, because it has longer side chain, slower rate of oxidation and metabolism within plant tissues, and can be converted to IAA (Strader and Bartel, 2011). Besides, NAA concentrations higher than the optimum stimulate ethylene biosynthesis affecting adversely rooting (Tesfa et al., 2016), thus enabling IBA to maintain the auxin activity in the medium for a longer period and promoting rooting better than NAA.

Table 1
Disinfection protocols tested on *Alkanna tinctoria* explants.

Type of explant	Season	Disinfection protocols			Number of explants	Disinfection success (%)
		Fungicide[a]	Ethanol[b]	NaOCl[c]		
Shoot-tip	Spring	+	–	1 (3 min)	75	22
Shoot-tip	Summer	+	+	2 (10 min)	25	25
Shoot-tip	Early-autumn	+	+	3 (10 min)	76	12
Shoot-tip	Late-autumn	+	+	3 (10 min)	25	47
Shoot-tip	Autumn	+	+	3 (15 min)	23	0
Nodes	Spring	+	+	–	51	0
Nodes	Spring	+	+	3 (15 min)	138	4

[a] Signum® fungicide at 0.07 g/100 mL ddH_2O for 30 min immersion.
[b] Immersion in ethanol for 1 min.
[c] NaOCl solutions at different concentrations (1, 2, 3%).

Table 2
Effects of Murashige and Skoog-MS, Woody Plant Medium-WPM, and Gamborg B5-GB5 culture media enriched with 1.1 μM BA, 0.15 μM NAA, 0.3 μM GA_3, and solidified with two gelling agents (6 g L^{-1} Plant Agar or 3 g L^{-1} Gelrite), on proliferation parameters of *Alkanna tinctoria* explants measured after 15 and 20 days of culture in the first (I) and second (II) experiment, respectively.

Exp.	Treatments	Shoot formation (%)	Number of new shoots/explant	Shoot length (cm)	Proliferation rate	Hyperhydricity (%)
I	MS+Plant Agar	100.0 ± 0.0 a	5.4 ± 0.4 a	1.4 ± 0.1 a	3.9 ± 0.4 a	15.0 ± 9.2 a
	WPM+Plant Agar	95.0 ± 5.0 a	3.5 ± 0.3 b	1.3 ± 0.1 a	2.7 ± 0.3 b	30.0 ± 5.8 a
	GB5+Plant Agar	95.0 ± 5.0 a	3.4 ± 0.3 b	1.3 ± 0.1 a	2.4 ± 0.2 b	15.0 ± 9.6 a
II	MS+Plant Agar	100.0 ± 0.0 a	4.7 ± 0.5 a	1.8 ± 0.1 a	4.2 ± 0.3 a	20.0 ± 8.2 a
	WPM+Plant Agar	75.0 ± 5.0 b	3.5 ± 0.4 b	1.4 ± 0.1 b	2.8 ± 0.3 c	20.0 ± 8.2 a
	MS+Gelrite	100.0 ± 0.0 a	4.7 ± 0.4 a	1.5 ± 0.1 b	3.8 ± 0.3 ab	20.0 ± 14.1 a
	WPM+Gelrite	95.0 ± 5.0 a	4.6 ± 0.3 ab	1.4 ± 0.1 b	3.3 ± 0.2 bc	25.0 ± 12.6 a

Data for number of new shoots per explant, shoot length, and proliferation rate are means ± S.E. of 20 replicates, while data for shoot formation and hyperhydricity are means ± S.E. of 4 replicates (*i.e.*, the vessels, each containing 5 explants). For both experiments separately (I and II) means followed by different letters within the same column differed significantly (Duncan's multiple range test, $P \leq 0.05$).

Table 3
Effect of Murashige and Skoog-MS and ½ MS supplemented with three IBA concentrations, of MS with six NAA+IBA combinations, and of Root Culture 1-RC1 with four IAA concentrations, on rooting parameters of *Alkanna tinctoria* explants measured after 20 days of culture in the first (I) and second (II) experiments, and after 30 days in the third (III) experiment.

Exp.	Culture media	Treatments (μM)	Rooting (%)	Root number/ rooted explant	Root length (cm)	Hyperhydricity (%)
I	MS	0 IBA	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	20.0 ± 8.2 a
	MS	2.5 IBA	30.0 ± 5.8 a	3.3 ± 1.2 a	0.9 ± 0.1 a	10.0 ± 5.8 a
	MS	5 IBA	50.0 ± 17.3 a	3.2 ± 0.9 a	0.9 ± 0.1 a	15.0 ± 9.6 a
	½ MS	0 IBA	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	10.0 ± 10.0 a
	½ MS	2.5 IBA	45.0 ± 15.0 a	1.8 ± 0.4 a	0.6 ± 0.1 a	0.0 ± 0.0
	½ MS	5 IBA	25.0 ± 12.6 a	2.8 ± 1.1 a	0.7 ± 0.1 a	0.0 ± 0.0
II	MS	2.69 NAA + 0 IBA	6.7 ± 6.7 a	1.0 ± 0.0 d	0.5 ± 0.0c	6.7 ± 6.7 b
	MS	5.4 NAA + 0 IBA	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	13.3 ± 6.7 ab
	MS	2.69 NAA + 2.5 IBA	6.7 ± 6.7 a	3.0 ± 0.0 a	0.3 ± 0.0 d	13.3 ± 6.7 ab
	MS	5.4 NAA + 2.5 IBA	13.3 ± 6.7 a	1.5 ± 0.0 b	1.0 ± 0.1 b	13.3 ± 6.7 ab
	MS	2.69 NAA + 5 IBA	26.7 ± 17.6 a	1.0 ± 0.0 d	1.2 ± 0.1 a	13.3 ± 13.3 ab
	MS	5.4 NAA + 5 IBA	20.0 ± 0.0 a	1.3 ± 0.1 c	0.5 ± 0.0 c	46.7 ± 17.6 a
III	RC1	0 IAA	30.0 ± 5.8 b	5.1 ± 0.2 b	2.1 ± 0.3 b	0.0 ± 0.0
	RC1	2.85 IAA	80.0 ± 8.2 a	6.0 ± 0.8 a	3.4 ± 0.8 a	0.0 ± 0.0
	RC1	5.71 IAA	10.0 ± 5.8 c	1.0 ± 0.3 c	0.7 ± 0.2 c	0.0 ± 0.0
	RC1	11.42 IAA	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Data for root number per rooted explant and root length are means ± S.E. of 20 replicates for IBA and IAA treatments, and 15 for NAA+IBA combinations, while data for rooting and hyperhydricity are means ± S.E. of 4 replicates for IBA and IAA treatments, and 3 for NAA+IBA combinations (*i.e.*, the vessels, each containing 5 explants). For the experiments separately (I, II, and III), means followed by different letters within the same column differed significantly (Duncan's multiple range test, $P \leq 0.05$). Values in columns with means ± S.E. of 0 were not considered in the statistical analysis.

Indeed, these three auxin types have different receptors, uptake rates, transport, and metabolism (De Klerk et al., 1997) depending also on the concentration of the mineral elements presented in the medium (Shohael et al., 2013). Hyperhydricity was noticed in almost all treatments and in higher extent (46.7%) in *A. tinctoria* explants growing in MS + 5.4 μM NAA + 5 μM IBA (Table 3.I and II).

Interestingly, rooting percentage, root number, and root length were significantly higher in RC1 medium enriched with 2.85 μM IAA as compared with the other RC1 treatments (Table 3.III). Moreover, hyperhydricity symptoms were completely reversed after a 30-days culture period. According to Hartmann et al. (2011), IAA is the naturally occurring auxin produced in leaves and buds, and transported basipetally from the top towards the lower part of the plant (*i.e.*, base of the cut), thus promoting rooting through interactions with other endogenous substances that lead to its raised endogenous level. Hyperhydricity is reported to decline or totally reverse when using culture media with reduced or null concentrations of NH_4NO_3 (Liu et al., 2003), while NH_4NO_3 stimulated rejuvenation and height of *Paeonia lactiflora* Pall. hyperhydric explants (Wu et al., 2011). The irreversible loss of regenerative ability of tissues and the poor *ex vitro* survival rate of hyperhydric shoots limit the potential for *in vitro* mass propagation (Ivanova and van Staden, 2008).

The use of IAA and the elimination of NH_4NO_3 for *in vitro* rooting of *A. tinctoria* led to a successful rooting and further acclimatization protocol. In fact, the best *ex vitro* plant survival (100%) was obtained once applying explants rooted in RC1 + 2.85 μM IAA medium (Supplementary Table S3). Phulwaria and Shekhawat (2013), described a lower survival rate (60%) of *in vitro* rooted *A. hispidissima*, another important Alkannin yielding plant, while a higher rate (75%) of plants developed from *ex vitro* rooting. Moreover, this technique reduces the steps of micropropagation, saving cost, labor, and resources. In the present study, the asexual propagation by softwood cuttings gave a rooting percentage around 92%. This alternative, characterized by a direct *ex vitro* cultivation, could be taken into account in case no contaminant-free explants are needed in further applications, but it will not fulfill the need for a large-scale production of *A. tinctoria*. Nevertheless, the probable reason of higher survival percentages of *A. tinctoria* plants developed from *in vitro* rooting in the current study may be due to the fully developed root system (*i.e.*, more and longer lateral roots), enabling better adaptation to the acclimatization process. Similar results with high greenhouse survival rates were reported for *R. umbellate* (70–100%) (Perić et al., 2012) and *C. verbenacea* (90–95%) (Lameira and Pinto, 2006).

4. Conclusion

In conclusion, an efficient protocol for the micropropagation and *in vitro* rooting of an important medicinal plant, *A. tinctoria*, has been developed. High amounts of *in vitro* produced plants with high levels of survival during *ex vitro* acclimatization have been obtained. This could presumably reduce the pressure on the natural population of this plant and at the same time open the door for mass-production of *in vitro* or

acclimatized plants useful for the production of SMs. Plant tissue culture represents thus a useful tool for the conservation and propagation of this important medicinal plant, potentially appropriate for the large-scale production of A/S *via* root induction either *in vitro* and/or growth under *ex vitro* conditions.

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CRediT authorship contribution statement

Annalisa Cartabia: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. **Virginia Sarropoulou**: Data curation, Formal analysis, Investigation, Writing – original draft. **Katerina Grigoriadou**: Conceptualization, Funding acquisition, Methodology, Project administration, Writing – review & editing. **Eleni Maloupa**: Funding acquisition, Project administration, Supervision. **Stéphane Declerck**: Funding acquisition, Supervision, Project administration, Writing – review & editing. All authors approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2022.114860.

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