Eur. J. Hortic. Sci. | ISSN 1611-4426 print, 1611-4434 online | https://doi.org/10.17660/eJHS.2022/017 | © ISHS 2022

Original article

Vegetative and *in vitro* propagation of the medicinal and ornamental plant *Astragalus suberosus* subsp. *haarbachii* (Fabaceae)

K. Grigoriadou, V. Sarropoulou, N. Krigas and E. Maloupa

Hellenic Agricultural Organization (HAO)-DEMETER, Institute of Plant Breeding and Genetic Resources, Laboratory of Protection and Evaluation of Native and Floriculture Species, Balkan Botanic Garden of Kroussia, Thessaloniki, Greece

Summary

A number of *Astragalus* species (Fabaceae) are utilized for medicinal (secondary metabolites) and ornamental purposes (landscaping), and in food industry for the production of high quality forages and gum. However, the slow seedling development, the poor seed germination capacity and low number of seed set are often drawbacks for their propagation. According to the literature, there is no prior information on the asexual propagation of the Balkan sub-endemic *A. suberosus* subsp. *haarbachii*. Propagation experiments were performed to set up a protocol for the vegetative and *in vitro* propagation of this taxon, suitable at nursery level. In early spring, the base of softwood tip cuttings (7–8 cm) was immersed for 1 min in IBA (0, 1,000, 2,000, 4,000 ppm) solutions and overlapped with 0.066% and 0.2% powdered IBA formula. The cuttings were placed in trays on a peat: perlite (1:3) substrate on a heated greenhouse mist. The most appropriate rooting treatment was the 2,000 ppm IBA (17.3 roots per cutting, 2.7 cm long, 100% rooting) in 5 weeks and the plants' survival rate after 10 weeks was 81.3–100%. Among the three basal culture media (MS, WPM, Gamborg B5) tested *in vitro*, B5 gave the highest shoot formation percentage (62.5%) and leaf length, MS and B5 gave similar shoot numbers while the percentage of explants' with yellowish leaves was lower in MS. The combined effect of 8 µM BA + 0.2 µM IBA (MS medium) gave the highest shoot formation (66.7%), shoot number (1.9) and length (0.9 cm) promoting initial shoot induction. This study contributes to the successful *ex situ* conservation of *A. suberosus* subsp. *haarbachii*, facilitating its further sustainable exploitation.

Keywords

Astragalus, auxins, conservation, cytokinins, rooting, tissue culture

Significance of this study

What is already known on this subject?

- *Astragalus suberosus* subsp. *haarbachii* is considered as medicinal (secondary metabolites), productive (high quality forages, gum) and ornamental plant (landscaping). There is no prior information for the propagation of *A. suberosus* subsp. *haarbachii*, either sexually through seeds or asexually through cuttings and *in vitro*, thus research is urgently needed.

What are the new findings?

- Asexual propagation protocols (cuttings, *in vitro*) of *A. suberosus* subsp. *haarbachii* were established for the first time. IBA concentration and application method affect rooting of cuttings (5 weeks, spring) being 100% with 2,000 ppm IBA (1 min). An *in vitro* shoot proliferation protocol is provided, based on different basal culture media (MS, WPM, B5) and PGR's concentrations, being optimum (66.67%) in MS medium with 8 µM BA + 0.2 µM IBA.

What is the expected impact on horticulture?

- Asexual propagation protocols were developed for possible reinforcement of wild populations *A. suberosus* subsp. *haarbachii* in the natural environment and for gardening or landscaping applications, speeding up production for growers. The *in vitro* plantlets may be suitable to set up a transformation system for the production of transgenic plants with desirable horticultural traits or medicinal contents.

Introduction

Many species and subspecies of the genus *Astragalus* (Fabaceae) are medicinal plants due to their secondary metabolites such as polysaccharides, saponins and isoflavonoids (Tang, 1992; Bedir et al., 2000; Karagöz et al., 2007) and are used as hepatoprotective, antioxidative, antidiabetic, antineoplastic, immune-stimulant and antiviral (Bedir et al., 2000; Erisen et al., 2010b) or for gum production (Rios and Waterman, 1997). Additionally, they are used for the production of high quality forages for animal nutrition (Bedir et al., 2000; Cho et al., 2000) and they are also used for arid landscaping and erosion control through establishment of rangelands and artificial meadows stabilised due to their deep top rooting system (Erisen et al., 2010a).

Slow seedling development, poor seed germination capacity and low number of seed set in species of *Astragalus* are usual drawbacks affecting their wider utilization (Yorgancilar and Erisen, 2011). Although vegetative propagation is the most popular method used in commercial nurseries to propagate plants of the Fabaceae family including *Astragalus* spp. (Hrynkiewicz-Sudnik et al., 2001; Hartmann et al., 2011), several studies have been conducted regarding the *in vitro* culture and mass multiplication of *A. trigo-*

nus for germplasm preservation (Vicente et al., 2011) or for the adventitious shoot regeneration via organogenesis and embryogenesis studied in *A. adsurgens* Pall. (Luo and Jia, 1998a, b), *A. cicer* L. (Uranbey et al., 2003; Başalma et al., 2008), *A. melilotoides* Pall. (Hou and Jia, 2004), *A. polemoniacus* Bunge (Mirici, 2004), *A. chrysochlorus* Boiss. & Kotschy (Turgut-Kara and Arı, 2008), *A. cariensis* Boiss. (Erisen et al., 2010a) and *A. nezaketae* A. Duran & Aytaç. (Erisen et al., 2010b). However, there is limited research to date concerning the *in vitro* multiplication of *Astragalus* spp., which is mostly confined to *A. duranii* E. Hamzaoğlu & L. Kurtis (Çöçü et al., 2005), *A. maximus* Willd. (Turgut-Kara and Arı, 2006) and *A. nitidiflorus* Jiménez & Pau (Cano-Castillo et al., 2009).

Astragalus suberosus subsp. *haarbachii* (Boiss.) V.A. Matthews (including the indistinguishable *A. maniaticus* Kit Tan & Strid, see Kougioumoutzis et al., 2017) is a Balkan-centered subspecies, extending to Turkey, that grows on agricultural and ruderal lowland habitats (Dimopoulos et al., 2013, 2016), surviving harsh environmental conditions as seeds (therophyte) or with perennating buds at ground level protected by leaf or stem bases (hemicryptophyte). The dual life-form (annual or short-lived perennial) of *A. suberosus* subsp. *haarbachii* and the fact that is adapted to withstand dry conditions and arid areas render it as eligible for ornamental landscaping with native plants. In this context, the vegetative propagation and *in vitro* culture of *A. suberosus* subsp. *haarbachii* was studied for the first time in an attempt to facilitate the *ex situ* conservation and sustainable utilization of this possibly medicinal and ornamental plant. This was done in the frame of implementation of the policy developed by the Balkan Botanic Garden of Kroussia (BBGK) focusing on propagation, evaluation, conservation and sustainable use of prioritised native plants of Greece and the Balkans, combined with actions raising the environmental awareness of the public (Krigas and Maloupa, 2008; Maloupa et al., 2008).

Materials and methods

Vegetative propagation of *A. suberosus* subsp. *haarbachii*

1. Plant material. Plant material of *A. maniaticus* which is indistinguishable from *A. suberosus* subsp. *haarbachii* (Kougioumoutzis et al., 2017) was collected from cultivated areas close to Prastos village in Arcadia prefecture, Peloponnese, South Greece, at an altitude of ca. 700 m (Figure 1A, B). The plant material was collected during botanic expeditions conducted with a special collection permit issued by the Greek Ministry of Environment and Energy. The plants collected were taxonomically identified as *A. suberosus* subsp. *haarbachii* (Kougioumoutzis et al., 2017), and they were given the unique International Plant Exchange Network (IPEN) accession number GR-1-BBGK-04,2642. The plants received immediate care in the BBGK's nursery to recover from transplantation shock, being placed in partial shade within the well-aerated greenhouse prior to further experimentation.

2. Vegetative propagation. In spring (middle March), softwood tip cuttings of *A. suberosus* subsp. *haarbachii* were cut from mother plants originating from the wild which were initially developed inside greenhouse conditions. The upper part of the cutting was used. The cutting position on shoot was terminal. The mean length of cuttings was 7–8 cm and the mean number of leaves per cutting was 4. The base of the cuttings was immersed for 1 min in IBA (0, 1,000, 2,000 and 4,000 ppm) solutions and overlapped with powdered-form IBA (commercial formulation: Radicin containing 0.066% and 0.2% IBA). The solvent used for IBA solutions was ethanol by dissolving IBA in 97% ethanol and diluting to a final volume of 50% ethanol and filling the remaining 50% of the volume with water. The pure form of IBA was used, not its salt formulation, for both solutions and powdered form under the commercial formula Radicin. Afterwards, the cuttings were placed in propagation multi-position trays (12×7=84) with dimensions of 50 cm × 30 cm in diameter, and 178 cm^3 or mL soil volume per planting unit in a substrate mixture consisted of peat (Terrahum, Klasmann) and perlite (Geoflor) (1:3 v/v) and maintained at bottom-heated benches in BBGK's greenhouse covered with plastic. Medium soil temperature was kept at 18–21°C while air temperature was 15–25°C, depending on weather conditions. Relative humidity (RH) was approximately 70–85% (mist). At the end of the experiment period (5 weeks), the number of roots per rooted cutting and root length were measured. Rooting was expressed as percentage (%). Produced rooted plants were then transplanted in pots of 0.33 L (8×8×7 cm) containing a mixture of peat (Klasmann, TS2), perlite and soil (2:½:½ v/v ratio) and subsequently after 40 days in pots of 2.5 L containing a mixture of peat (Klasmann, TS2), perlite and soil (2:1:1 v/v ratio). The survival percentage in transplantations of rooted cuttings into pots of larger capacity was recorded.

In early-June, after the end of experimentation and the recording of the final survival percentage of the generated plants outdoors of the sea level greenhouse in Thermi (metropolitan Thessaloniki), a number of plants was transplanted for long-term *ex situ* conservation and monitoring in plant displays at the grounds of the BBGK, in Kilkis prefecture (Northern Greece), at an altitude of 650 m a.s.l. (Krigas and Maloupa, 2008).



Figure 1. Plant material of *Astragalus suberosus* subsp. *haarbachii*, whole plant collected from the wild, South Greece, altitude of ca. 700 m, at the stage of stem vegetative growth and full flowering (A), inflorescence with flower in magnification (B), and initial establishment and *in vitro* shoot induction after a 40-day culture period in MS medium supplemented with 1.5 μM BA, 0.15 μM IBA, 20 g L^{-1} sucrose and solidified with 6.5 g L^{-1} agar (pH 5.8) (C).

In vitro shoot proliferation of *A. suberosus* subsp. *haarbachii*

1. Disinfection and establishment of plant material *in vitro*.
Winter trials

During mid-winter (December), 6 pots of mother plants of *A. suberosus* subsp. *haarbachii* collected from the wild were transferred from the external environment to the greenhouse. After a 15-day period of adaptation under controlled conditions (8–17°C, RH 50–65%), the upper part of shoots with two buds was removed from mother plants, dissected in smaller segments and used as initial explants. Prior to disinfection, the explants were washed with tap water and then were soaked in 70% ethanol solution for 30 sec followed by 2% NaOCl solution for 20 min under agitation, and were rinsed three times with sterile distilled water. The large-sized leaves were removed from the base of the explants ($n = 73$, in total), and were cultured in the Murashige and Skoog (MS) basal nutrient medium (Murashige and Skoog, 1962) supplemented with 1.5 μM benzyladenine (BA), 0.15 μM IBA, 20 g L^{-1} sucrose and solidified with 6.5 g L^{-1} agar (pH 5.8). After 80 days of culture, the numbers of successful establishment, infected (bacteria and/or fungi) explants and browning/necrotic (due to disinfection toxicity) explants were recorded. Thereafter, the percentage of the successfully disinfected and established explants was calculated. Following that period, the explants were sub-cultured for six successive rounds in the above-mentioned medium at 30-day-intervals, accordingly, where the shoot proliferation rate was noted each time.

Spring trials

During early spring (March), the upper part of shoots with 1–2 buds was dissected and removed from the same mother plants maintained inside the greenhouse, serving as initial explants. Similar procedure was followed for disinfection as mentioned above. The base of the explants, the large-sized leaves close to base and the flowering buds were removed and the remaining part of explants ($n = 36$, in total) was cultured. After 80 days of culture and subsequently, the explants were sub-cultured for six successive rounds in two different media, i.e., (A): MS supplemented with, 1.5 μM BA, 0.15 μM IBA, 20 g L^{-1} sucrose and 6.5 g L^{-1} agar (pH 5.8), and (B): MS supplemented with 1.5 μM BA, 0.05 μM α-naphthalene acetic acid (NAA), 20 g L^{-1} sucrose and 6.5 g L^{-1} agar (pH 5.8) at 30-day-intervals, where the shoot proliferation rate (number of explants with production of new shoots/total number of initially explants used) was noted each time. After 80 days of culture, the percentages of both successfully established (pathogen-free) and infected (with fungi and/or bacteria) explants as well as the percentage of browning/necrotic explants due to disinfection toxicity were recorded.

All successfully established plant material was sub-cultured in MS (1.5 μM BA, 0.15 μM IBA) every 30 days and it was used for further experimentation.

2. *In vitro* shoot proliferation. The experimental plant material used for proliferation experiments was shoot-tip explants of 1–1.5 cm long obtained from stock *in vitro* cultures.

The effect of three different basal culture media was studied, i.e., MS (Murashige and Skoog, 1962), Woody Plant Medium (WPM) (Lloyd and McCown, 1980) and Gamborg B5 (Gamborg et al., 1968). All three basal media were supplemented with 1.5 μM BA, 0.15 μM IBA, 20 g L^{-1} sucrose and solidified with 6 g L^{-1} agar.

Furthermore, the effect of five different concentrations of the cytokinin BA (0, 1, 2, 4 and 8 μM, applied with 0.2 μM IBA) on shoot proliferation ability was studied. The control treatment consisted of 0.2 μM IBA (BA-free). The basal culture medium used was the MS supplemented with 20 g L^{-1} sucrose and solidified with 6 g L^{-1} agar.

In both experiments *in vitro*, 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. Shoot-tip explants were transferred into Magenta vessels containing 35 mL of MS medium. All cultures were maintained in a growth chamber with constant temperature of 22 ± 2°C and 16-h light photoperiod (40 μmol m^{-2} s^{-1}) supplied by cool white fluorescent lamps. After 30 days of culture, shoot formation percentage (%), number of shoots/explants, shoot length (cm), leaf length (cm) and percentage of explants with yellowish leaves appearance (%) were recorded.

Statistical analysis

Both *in vitro* experiments were completely randomized and analyzed with ANOVA (Analysis of Variance) using the statistical programme SPSS 17.0 (SPSS Inc., Illinois, New York, U.S.A.) at $P \leq 0.05$, according to Duncan's multiple range test ± standard error (S.E.) in order significant differences among treatments to be established. The experiments comprised of eight groups with five replicates each (8 Magenta vessels × 5 explants each) and they were repeated twice. All the experiments were completely randomized and analyzed with ANOVA using the statistical program SPSS 17.0 (SPSS Inc., Illinois, New York, U.S.A.). For means separation the Duncan's Multiple Range Test ± standard error (S.E.) was used and significance was recorded at $P \leq 0.05$. The factorial experiment related to the vegetative propagation via rooting of cuttings included 6 treatments, 3 replications and 14 cuttings (replicates) per treatment for each replication (3 groups of 14 cuttings per treatment), giving $3 \times 14 = 42$ in total cuttings per treatment. The main effect of factors (application form of IBA and IBA concentration) and their interaction was determined by the General Linear Model and 2-way ANOVA. The two *in vitro* shoot proliferation experiments were comprised of eight groups with five replicates each (8 Magenta vessels × 5 explants each, and they were repeated twice (one-way ANOVA), thus 2 times × 40 = 80 in total cuttings were used per treatment. The first *in vitro* shoot proliferation experiment was repeated twice and included three treatments related to the three different basal culture media (MS, WPM, Gamborg B5) with 40 explants per treatment (8 groups of 5 replicates) × 2 times = 80 explants in total per treatment. The second shoot proliferation experiment was repeated twice and included five treatments related to the five different concentrations of BA (0, 1, 2, 4, 8 μM) with 40 explants per treatment (8 groups of 5 replicates) × 2 times = 80 explants in total per treatment. Therefore, the series $8 \times 5 \times 2$ corresponding to 8 groups (vessels) × 5 explants × 2 times = 80 replicates (*in vitro* explants) were used in statistical analysis.

Results

Vegetative propagation of *A. suberosus* subsp. *haarbachii*

Regarding *A. suberosus* subsp. *haarbachii*'s softwood tip cuttings (spring, March), the best rooting treatment within five weeks was the 2,000 ppm IBA (17.29 roots 2.66 cm long, 100% rooting). IBA solutions showed better results compared to its powdered-form. More specifically, IBA solutions

raised rooting percentage (92.86%–100%) and root length (2.66–2.72 cm) whereas powder-form of IBA decreased rooting (71.43%–78.57%) with respect to the control (85.71%) (Table 1, Figure 2).

The survival percentage of rooted cuttings three weeks after their first transplantation into 0.33-L pots was 100% for the plants originated from 0, 1,000 and 2,000 ppm IBA treatments, and 92.86%, 90%, and 81.82% from plants originated from 4,000 ppm IBA, 0.066% IBA and 0.2% IBA, respectively. No further losses were observed six weeks after their subsequent transplantation from 0.33-L to 2.5-L pots, thus, the same survival percentages were recorded as previously mentioned. No differentiation in shoot growth and development of cuttings was observed, regardless concentration (0–4,000 ppm) and application form (solution/powder) of IBA (Figure 2).

All transplanted plants were successfully acclimatised and they were established in plant displays for long-term *ex situ* conservation facility of the BBGK, at an altitude of 650 m a.s.l.

Table 1. Effect of application type (solution, powder) and indole-3-butyric (IBA) concentration (solutions: 0, 1,000, 2,000, 4,000 ppm, and commercial powder Radicin: 0.066%, 0.2%) on rooting percentage (%), root number/rooted cutting and root length (cm) in *Astragalus suberosus* subsp. *haarbachii* softwood tip cuttings after 5 weeks in spring (16/3/2018–19/4/2018).

Treatments (IBA)	Rooting (%)	Root number	Root length (cm)
Control	85.71 ± 1.83 c	14.75 ± 2.06 bc	1.98 ± 0.23 b
1,000 ppm	92.86 ± 1.15 b	21.85 ± 2.70 a	2.72 ± 0.25 a
2,000 ppm	100.00 ± 0.00 a	17.29 ± 1.80 ab	2.66 ± 0.28 a
4,000 ppm	100.00 ± 0.00 a	15.07 ± 1.33 bc	2.69 ± 0.21 a
0.066%	71.43 ± 2.89 e	10.50 ± 1.19 cd	2.23 ± 0.18 ab
0.2%	78.57 ± 1.73 d	6.70 ± 1.11 d	1.77 ± 0.19 b
P-values (General Linear Model/ 2-way ANOVA)			
Application form of IBA (A)	0.000***	0.000***	0.014*
IBA concentration (B)	0.000***	0.071 ns	0.134 ns
(A)*(B)	0.000***	0.004**	0.148 ns

Means ± standard error (S.E.) with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$. ns: $P \geq 0.05$; *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$.

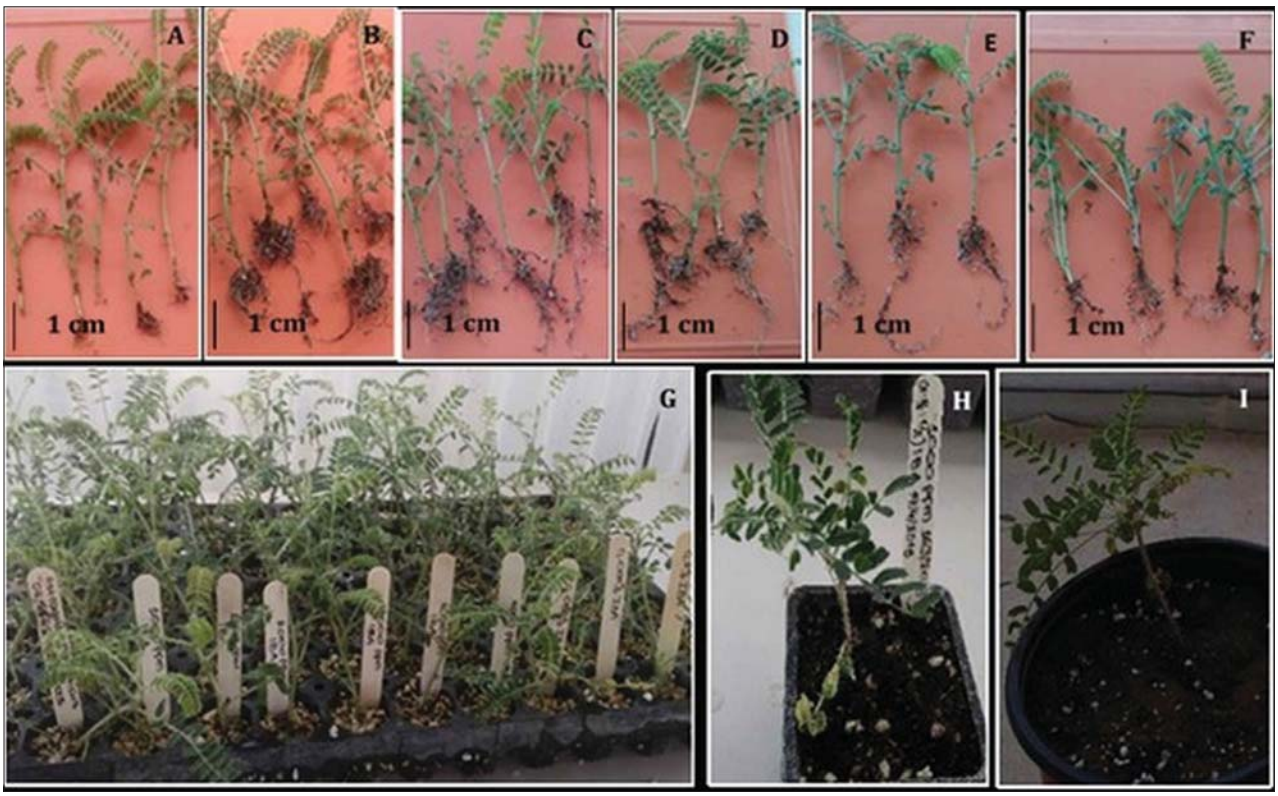


Figure 2. Asexual propagation of *Astragalus suberosus* subsp. *haarbachii* by softwood tip cuttings in spring (5 weeks), in the control IBA-free treatment (A), 1,000 ppm IBA (B), 2,000 ppm IBA (C), 4,000 ppm IBA (D), 0.066% IBA (E), 0.2% IBA (F), rooting of cuttings in multi-plate discs in all treatments (G), transplantation and vegetative growth of rooted cuttings into 0.33-L pots (H), and subsequently into 2.5-L pots (I).

In vitro shoot proliferation of *A. suberosus* subsp. *haarbachii*

1. Disinfection and establishment of plant material *in vitro*.

Winter trials

After the first 80 days of culture, only 2 out of the 73 initial explants were fungi- and bacteria-free, exhibiting 2.7% disinfection success. Following this period, the shoot proliferation rate of explants after six subsequent subcultures (30 days-intervals) in the medium of the initial establishment was in average 1.87; this finally resulted in 85 plantlets (Figure 1C).

Spring trials

In spring trial, after the first 80 days of culture, only 5 out of the initial 36 explants were fungi- and bacteria-free, exhibiting 13.5% disinfection success. Following this period, the shoot proliferation rate of explants after six subsequent subcultures (30 days-intervals) from the initial establishment was 1.38 at medium A and 1.37 at medium B. In spring trial, the percentage of successfully established explants due to disinfection increased to 13.5% compared to the 2.7% recorded in the winter trial.

2. *In vitro* shoot proliferation. Among the three basal culture media (MS, WPM, B5) used, the B5 medium exhibited the higher shoot formation percentage (62.5%), the WPM the lowest (20%), while 45% of the explants cultured in MS medium for 30 days led to the production of multiple shoots. MS and B5 media gave similar shoot numbers (1.63–1.80 shoots explant^{-1}) in relation to WPM (1.28 shoots explant^{-1}). The three different basal culture media did not show a significant effect on shoot length (1.07–1.48 cm). The percentage of explants with yellowish leaf appearance was lower (72.5–80%) and the length of leaves was higher (4.34–4.56 cm) with both MS and WPM. On the other hand, the highest leaf length and percentage of explants with yellowish leaves were observed in B5 medium. Taking concurrently all parameters into consideration, the WPM medium was the least effective, B5 although recording higher shoot formation percentage eventually led to yellowish leaf appearance of all explants, while the MS medium was proved to be the most appropriate one (Table 2, Figure 3).

The combined effect of 8 µM BA with 0.2 µM IBA in MS medium gave better results in terms of shoot formation percentage (66.67%), shoot number (1.87 shoots explant^{-1}) and shoot length (0.9 cm) compared to the control BA-free treatment (6.67% shoot formation, 0.97 shoots 0.67 cm high).

Table 2. Effect of 3 different basal culture media (MS, WPM, Gamborg B5) supplemented with 1.5 µM benzyladenine (BA), 0.15 µM indole-3-butyric acid (IBA), 20 g L^{-1} sucrose and 6 g L^{-1} agar (pH 5.8) on shoot formation percentage (%), shoot number explant^{-1}, shoot length (cm), leaf length (cm) and percentage of explants with yellowish leaf appearance in *Astragalus suberosus* subsp. *haarbachii* shoot-tip explants after 30 days of *in vitro* culture.

Basal culture media	Shoot formation (%)	Shoot number	Shoot length (cm)	Leaf length (cm)	Explants with yellowish leaves (%)
MS	45.0 ± 5.00 b	1.63 ± 0.12 a	1.48 ± 0.09 a	4.56 ± 0.20 a	72.5 ± 7.15 b
WPM	20.0 ± 5.35 c	1.28 ± 0.09 b	1.42 ± 0.10 a	4.34 ± 0.24 a	80 ± 6.40 b
Gamborg B5	62.5 ± 7.01 a	1.80 ± 0.11 a	1.07 ± 0.07 a	3.73 ± 0.16 b	100 ± 0.00 a
P-values	0.000***	0.004**	0.002**	0.013*	0.002**

Means ± standard error (S.E.) with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at P ≤ 0.05. *: P ≤ 0.05; **: P ≤ 0.01; ***: P ≤ 0.001.

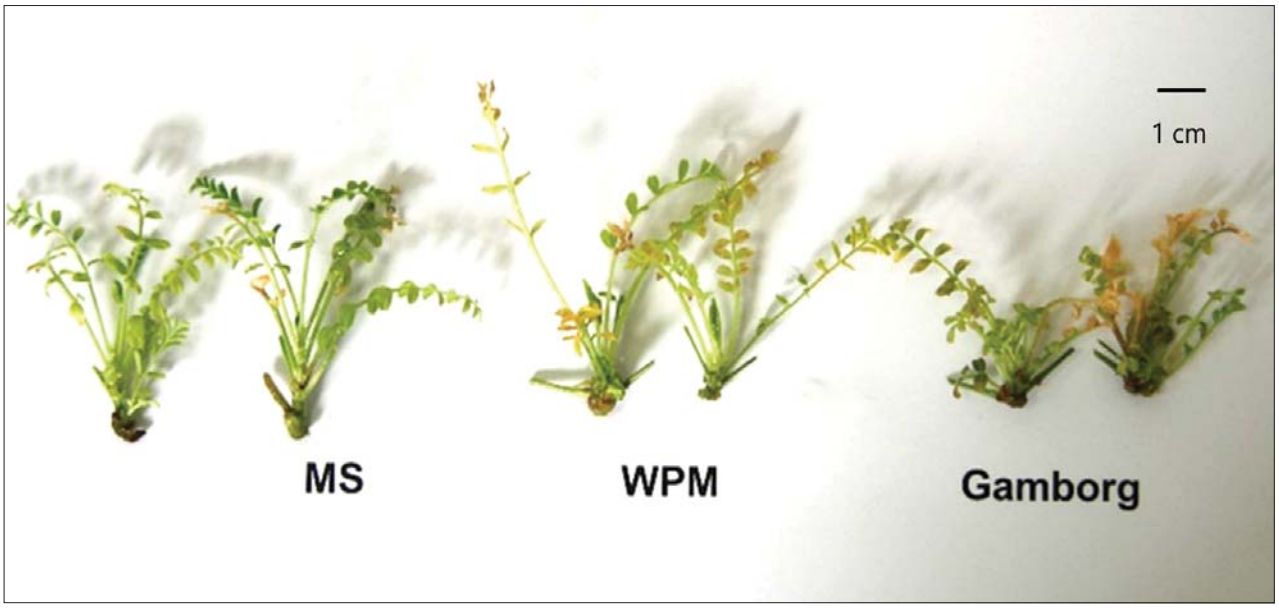


Figure 3. Effect of 3 different basal culture media (MS, WPM, Gamborg B5) supplemented with 1.5 µM BA, 0.15 µM IBA, 20 g L^{-1} sucrose and 6 g L^{-1} agar (pH 5.8) on shoot proliferation of *Astragalus suberosus* subsp. *haarbachii* explants after 30 days of *in vitro* culture.

The highest applied BA concentration (8 μM) caused a 10-fold increase in shoot formation (%), doubled the number of shoots and raised shoot length by 30% with respect to the control. However, the length of leaves was highest (6 cm) in the absence of BA from culture medium (control), thus, the increase in BA concentration led to the decrease of leaf length of explants (2.75–4.23 cm). In all five treatments, explants with yellowish leaves were evident (14.81–48.15%); this was less intense though in the case of BA-untreated explants (14.81%). Among the BA-treated explants, no substantial differences were observed regarding the percentage of explants with yellowish leaves (35.71–48.15%) (Table 3, Figure 4).

Discussion

Vegetative propagation of *A. suberosus* subsp. *haarbachii*

To the best knowledge of the authors, there is no prior information on the vegetative propagation of *A. suberosus* subsp. *haarbachii*. As revealed in the present study, its rooting was optimum with 2,000 ppm IBA for softwood tip cuttings in spring. According to Khanal et al. (2015), 4-hour dipping of *A. adsurgens* cuttings (hardwood, semi-hardwood and top shoot) in IAA, NAA, IBA solutions and their mixtures (100 ppm, each) exhibit similar results and high degree of effectiveness for plant survival, rooting percentage and av-

Table 3. Effect of benzyladenine (BA) concentration (0, 1, 2, 4, 8 μM) applied in combination with 0.2 μM indole-3-butyric acid (IBA) on shoot formation percentage (%), shoot number explant^{-1}, shoot length (cm), leaf length (cm) and percentage of explants with yellowish leaf appearance in *Astragalus suberosus* subsp. *haarbachii* explants after 30 days of *in vitro* culture in MS medium containing 20 g L^{-1} sucrose and 6 g L^{-1} agar (pH 5.8).

BA (μM)	Shoot formation (%)	Shoot number	Shoot length (cm)	Leaf length (cm)	Explants with yellowish leaves (%)
0	6.67 ± 4.22 c	0.97 ± 0.08 c	0.69 ± 0.06 b	6.00 ± 0.21 a	14.81 ± 6.97 b
1	16.67 ± 6.15 c	1.17 ± 0.10 c	0.90 ± 0.07 a	4.23 ± 0.20 b	41.38 ± 9.31 ab
2	40.00 ± 2.58 b	1.33 ± 0.13 bc	0.87 ± 0.06 ab	3.84 ± 0.20 b	48.15 ± 9.80 a
4	40.00 ± 12.65 b	1.57 ± 0.17 ab	0.85 ± 0.07 ab	3.07 ± 0.18 c	35.71 ± 9.22 ab
8	66.67 ± 4.22 a	1.87 ± 0.16 a	0.90 ± 0.07 a	2.75 ± 0.11 c	37.93 ± 9.17 ab
P-values	0.000***	0.000***	0.136 ns	0.000***	0.116 ns

Means ± standard error (S.E.) with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at P ≤ 0.05. ns: P ≥ 0.05; ***: P ≤ 0.001.

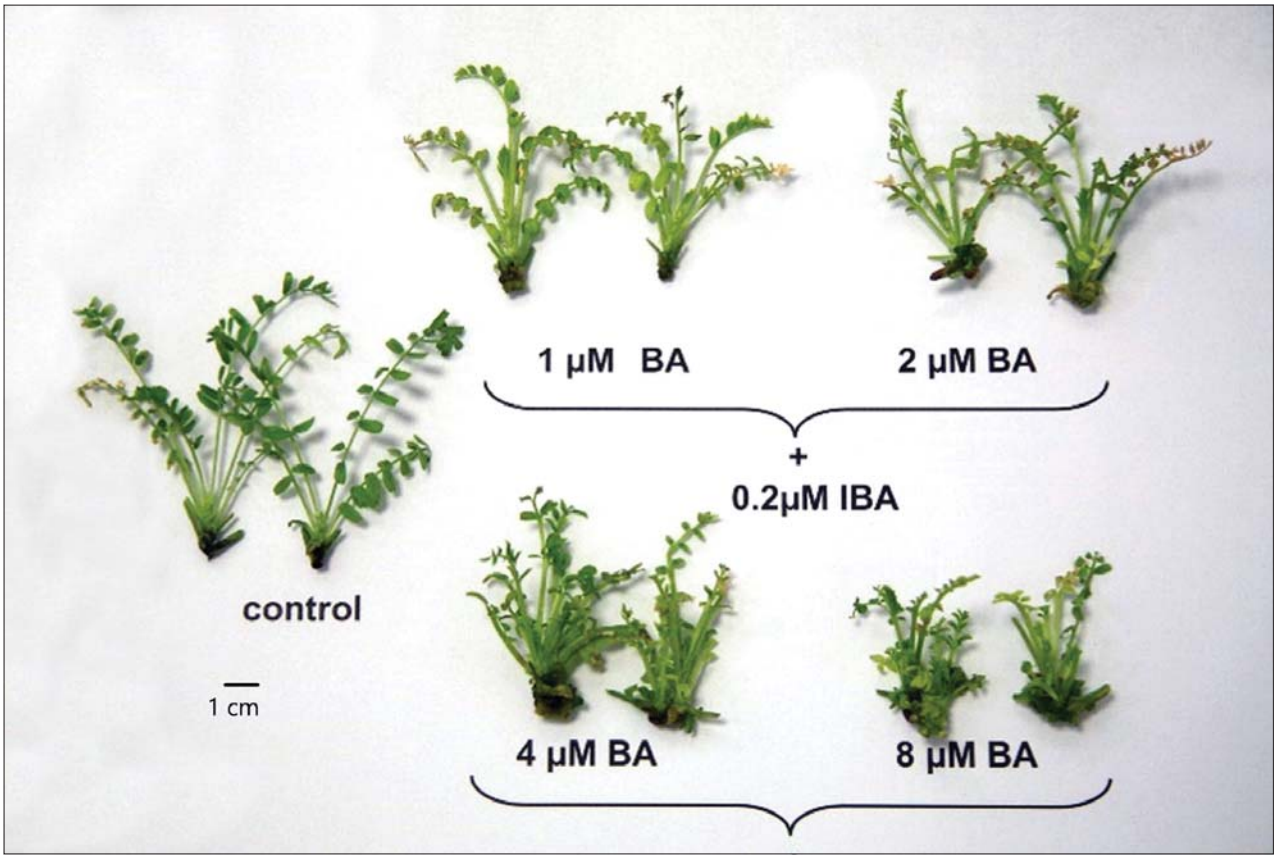


Figure 4. Effect of increasing BA concentration (0, 1, 2, 4, 8 μM) applied in combination with 0.2 μM IBA on shoot proliferation of *Astragalus suberosus* subsp. *haarbachii* explants after 30 days of *in vitro* culture [MS medium + 20 g L^{-1} sucrose + 6 g L^{-1} agar (pH 5.8)].

erage root length after a 4-week period. In the current study involving *A. suberosus* subsp. *haarbachii'*s softwood tip cuttings, the application form of IBA proved to be an important factor for rooting success, since IBA solutions irrespective of concentration were more effective than the powdered-form of IBA. The above results show that auxins improved the rooting of the studied taxon. On the contrary, different results to ours are reported for other *Astragalus* species such as *A. membranaceus* Bunge var. *alpinus* N.; in the latter, the *in vivo* rooting rate of the cuttings was induced up to 65% by 0.5% IBA/Talc powder treatment, however the highest applied concentration of 2% powdered IBA had an inhibitory effect (Han et al., 2014).

In vitro shoot proliferation of *A. suberosus* subsp. *haarbachii*

Very few plant regeneration systems have been reported for *Astragalus* spp. to date. Literature review shows that *Astragalus* plants can be regenerated either from subterranean parts such as hairy roots (Cho et al., 1998), or aerial parts such as axillary buds (Turgut-Kara and Ari, 2006), leaves and leaf petioles (Uranbey et al., 2003; Mirici, 2004; Erisen et al., 2010a, b), or from embryonic parts such as hypocotyls and cotyledons (Basalma et al., 2008), calli and protoplasts (Luo and Jia, 1998a, b), and somatic embryos (Luo et al., 1999; Hou and Jia, 2004; Turgut-Kara and Ari, 2008). In the study involving *A. suberosus* subsp. *haarbachii,* shoot tips were used as the only explant type, and among the different basal culture media (MS, WPM, B5) tested, the MS medium supplemented with 1.5 μM BA, 0.15 μM IBA, 20 g L^{-1} sucrose and 6 g L^{-1} agar proved to be the most effective for *in vitro* shoot proliferation of shoot tip explants with 1–2 buds (Experiment No. 1). Luo and Jia (1998b), Luo et al. (1999), Hou and Jia (2004) also report that the medium selected plays an important role in inducing shoot formation in other *Astragalus* species. In line with our findings, the MS medium fortified with 2.22 μM BA, 2.69 μM NAA, 30 g L^{-1} sucrose and 8 g L^{-1} agar provide better results with respect to the number of shoots (14 shoots $explant^{-1}$) and the length of shoots (10 cm) in *A. trigonus* explants (Sief-Eldein et al., 2018). Similarly, according to Cano-Castillo et al. (2009), the best proliferation in *A. nitidiflorus* plant can be achieved with 0.44 μM BA. Different results to ours have been reported for the perennial vulneraria milkvetch (*A. vulnerariae* DC.), in which no shoot regeneration was noted on cotyledon leaf and shoot nodes using MS medium containing various concentration of BAP + NAA (Dilaver et al., 2017). In this study, the average number of *A. suberosus* subsp. *haarbachii* shoots ranged from 1.28 (WPM) to 1.8 (B5), while explants cultured in MS medium yielded 1.63 average shoot number. Our findings are up to a degree in agreement with those reported in four threatened North American *Astragalus* species (*A. columbianus, A. amblytropis, A. aquilonius* and *A. mulfordae* M. E. Jones), where average multiplication rates (number of shoots) of axillary microshoots can range from 2.5 (*A. aquilonius*) to 3.1 (*A. mulfordae*), after culture in MS medium with 0.44 μM BA (Edson et al., 1998).

BA is a commonly used cytokinin for *in vitro* culture of *Astragalus* species such as *A. adsurgens* (Luo and Jia, 1998a, b), *A. melilotoides* (Hou and Jia, 2004), *A. aquilonius* Barne, *A. amblytropis* Barne, *A. columbianus* Barne (Edson et al., 1994), and *A. cariensis* (Erisen et al., 2010a). With respect to the studied *A. suberosus* subsp. *haarbachii,* the MS basal medium used was found to be the most effective one, and BA was the selected cytokinin type applied at different concentrations in order to find its optimum concentration for maximum results. In other *Astragalus* species to date, different cytokinin types are found to be more effective in stimulating adventitious shoots regeneration of explants such as TDZ in *A. cicer* (Başalma et al., 2008), *A. duranii* (Çöcü et al., 2005) and *A. polemoniacus* (Mirici, 2004), whereas in *A. nezaketae* no shoot regeneration was reported from petiole and leaf explants cultured on MS medium containing TDZ (Erisen et al., 2010b). Another cytokine type, zeatin riboside (ZR), may enhance shoot regeneration of *A. maximus* explants to a higher degree when added at 2.28 μM in the MS medium (Turgut-Kara and Ari, 2006). In *A. membranaceus* var. *alpinus,* no distinct effect was found during the multiple shoot induction stage, between BA and kinetin treatment, each applied separately at a 0.1–5 mg L^{-1} concentration range, BA especially at 2 mg L^{-1} more enhanced the number of internodes, whereas kinetin specifically at 0.5 mg L^{-1} stimulated slightly further shoot elongation (Han et al., 2014).

In the present study with *A. suberosus* subsp. *haarbachii*, the addition of BA (1–8 μM) to the MS medium supplemented with 0.2 μM IBA positively influenced shoot proliferation of explants. Similar findings were obtained for another *Astragalus* species (*A. vulnerariae*), involving epicotyl explants cultured in MS medium fortified with 4.44 μM BA + 2.69 M NAA which exhibit 50% shoot formation with 3 $shoots\ explant^{-1}$ (Dilaver et al., 2017). However, when different type of explants (hypocotyls) was used, other cytokinin types such as kinetin applied at 2.32 μM in combination with 2.69 μM NAA proved to be more effective in shoot proliferation (86.67% shoot formation, 4.47 $shoots\ explant^{-1}$) for *A. vulnerariae* (Dilaver et al., 2017).

The percentage of *A. suberosus* subsp. *haarbachii* explants with multiple shoot production was better enhanced in the 8 μM BA + 0.2 μM IBA combination treatment. In other *Astragalus* species including *A. schizopterus* Boiss. (Yorgancilar and Erisen, 2011) and *A. adscendens* Boiss. & Hausskn. (Esmaeili et al., 2016), the highest number of shoots can be obtained in MS medium containing 4.4 μM BA, which is in agreement with our findings. The maximum number of shoots, increased shoot length and highest shoot formation (%) of *A. suberosus* subsp. *haarbachii* explants was observed when BA was added to the medium in its highest concentration (8 μM). Esmaeili et al. (2016) report that the highest numbers of shoots in *A. adscendens* can be found in the BA containing media, with the reduction of shoot length being observed as a negative effect of BA on shoot regeneration. The same authors report that no proliferation of *A. adscendens* explants can be detected in a medium without auxin and cytokinin (Esmaeili et al., 2016), pointing out the essential role of supplementing the nutrient culture medium with plant growth regulators (cytokinins + auxins) for the promotion of *Astragalus* explants' proliferation.

Conclusion

Softwood tip cuttings represent the appropriate starting material for the vegetative propagation of the Balkan sub-endemic *A. suberosus* subsp. *haarbachii*. Successful rooting 100% was achieved with the application of 2,000 ppm IBA in spring (5 weeks). The procedure to immerse cuttings in a 2,000 or 4,000 ppm IBA solution for 1 min was time-efficient and easy to perform. This study concludes that different basal culture media as well as different concentrations and combinations of plant growth regulators (PGRs) had significant consequences for the *in vitro* shoot proliferation of *A. suberosus* subsp. *haarbachii*, i.e., MS basal medium supple-

mented with 8 µM BA, 0.2 µM IBA for initial shoot induction and subsequent elongation of the produced multiple shoots in 30 days. This study reports the successful asexual propagation of *A. suberosus* subsp. *haarbachii* and delivers for the first time effective protocols that have been produced for its *ex situ* conservation at the Balkan Botanic Garden of Kroussia which can facilitate its further sustainable exploitation as a possibly medicinal plant with ornamental value. Production of this species-subspecies from cuttings utilizing its vegetative propagation could reduce the time to market compared to seed propagation and result in a more uniform (size) crop being more attractive for commercial ornamental nurseries to produce, ensuring that the species would become more widely available to the public for landscape use and for restoration projects. The protocol adopted for the clonal micro-propagation of this species should be used to create the bank of *in vitro* and live collections for introducing and reintroducing valuable genotypes and populations, as well as the plantation cultivating of species characterized by pronounced medicinal properties. Micropropagation (1) may produce clones that could be screened using bioassays to assure bioactivity; (2) allows the mass generation of plants with genetically identical chemotype for cultivation purposes; (3) proves useful to reduce consumption pressure on potentially threatened wild populations; (4) can provide an effective technique to those seeking to mold a supply chain of a product, in order to ensure the genetic homogeneity of plant clones, chemical profile, and finally sustainability of plants harvested in the wild; and (5) is an important tool for the propagation of selected lines in various breeding programs, as well as the recovery of pathogen-free material, or even for slow growth storage and the cryopreservation of valuable germplasm of the medicinal (secondary metabolites) and ornamental (landscaping) *A. suberosus* subsp. *haarbachii*. Micropropagation can be a part of a strategy to reinforce the supply, and quality of crops used for medicinal purposes and functional foods (high-quality forages and gum).

Acknowledgments

The postdoctoral research of Dr. V. Sarropoulou (scholarship holder) and the scientific publication were carried out within the framework "Strengthening of Postdoctoral Researchers" of the operational program "Development of Human Resources, Education and Lifelong Learning", 2014–2020, which was implemented from the National Scholarships Foundation (NSF) in Greece, and was co-funded by the European Social Fund and the Hellenic Republic.

References

Başalma, D., Uranbey, S., Gürlek, D., and Özcan, S. (2008). TDZ-induced plant regeneration in *Astragalus cicer* L. Afr. J. Biotechnol. *7*(8), 955–959. http://dx.doi.org/10.5897/AJB07.628.

Bedir, E., Çalış, I., Piacente, S., Pizza, C., and Khan, I.A. (2000). New flavonol glycoside from the aerial parts of *Astragalus vulneraria*. Chem. Pharm. Bull. *48*(12), 1994–1995. http://dx.doi.org/10.1248/cpb.48.1994.

Cano-Castillo, M., Serrano-Martínez, F., and Casas, J.L. (2009). *In vitro* propagation of *Astragalus nitidiflorus* (Leguminosae), an endemic and endangered species from South-East of Spain. Acta Hortic. *812*, 545–550. http://dx.doi.org/10.17660/ActaHortic.2009.812.79.

Cho, H.J., Brotherton, J.E., Song, H.S., and Widholm, J.M. (2000). Increasing tryptophan synthesis in a forage legume *Astragalus sinicus* by expressing the tobacco feedback-insensitive anthranilate synthase (ASA2) gene. Plant Physiol. *123*(3), 1069–1076. https://doi.org/10.1104/pp.123.3.1069.

Cho, H.J.J., Widholm, M., Tanaka, N., Nakanishi, Y., and Murooka, Y. (1998). *Agrobacterium rhizogenes*-mediated transformation and regeneration of the legume *Astragalus sinicus* (Chinese milk vetch). Plant Sci. *138*(1), 53–65. https://doi.org/10.1016/S0168-9452(98)00149-6.

Çöçü, S., Erişen, S., Duran A., Hamzaoğlu, E., Gürlek, D., Parmaksız, I., and Mirici, S. (2005). Lokal endemik *Astragalus duranii* (Fabaceae) türünün *in vitro* koşullarda hızlı çoğaltımı. 14'ü Biyoteknoloji Kongresi, 31 Ağustos–2 Eylül, Eskişehir, Turkey.

Dilaver, Z., Mirzapour, M., and Kendir, H. (2017). Breaking seed dormancy and micropropagation of perennial vulneraria milkvetch (*Astragalus vulnerariae* DC.). Acta Sci. Polon.-Horturum Cultus *16*(4), 79–88. https://doi.org/10.24326/asphc.2017.4.9.

Dimopoulos, P., Raus, Th., Bergmeier, E., Constantinidis, Th., Iatrou, G., Kokkini, S., Strid, A., and Tzanoudakis, D. (2013). Vascular Plants of Greece: An Annotated Checklist. Englera *3*, (Berlin-Dahlem, Germany: Botanic Garden and Botanical Museum, and Athens, Greece: Hellenic Botanical Society).

Dimopoulos, P., Raus, T., Bergmeier, E., Constantinidis, Th., Iatrou, G., Kokkini, S., Strid, A., and Tzanoudakis, D. (2016). Vascular plants of Greece – An annotated checklist. Suppl. Willdenowia *46*(3), 301–347. https://doi.org/10.3372/wi.46.46303.

Edson, J.L., Wenny, D.L., Leege-Brusven, A., Everett, R.L., and Henderson, D.M. (1994). Conserving Threatened Rare Plants: Some Nursery Strategies. National Proceedings, Forest and Conservation Nursery Associations (Fort Collins, CO, U.S.A.: USDA For. Serv., Rocky Mtn. Forest and Range Exp. Sta., Gen. Tech. Rep.) RM-GTR-257, p. 150–157.

Edson, J.L., Everett, R.L., Wenny, D.L., and Henderson, D.M. (1998). Shoot culture of *Astragalus*: Toward conserving a threatened genus. Botanic Gardens Micropropagation News *2*(3), 34–36.

Erisen, S., Yorgancılar, M., Atalay, E., and Babaoğlu, M. (2010a). Prolific shoot regeneration of *Astragalus cariensis* Boiss. Plant Cell Tiss. Organ Cult. *100*, 229–233. https://doi.org/10.1007/s11240-009-9638-3.

Erisen, S., Yorgancılar, M., Atalay, E., Babaoğlu, M., and Duran, A. (2010b). Callus induction and plant regeneration of the endemic *Astragalus nezaketae* in Turkey. Electron. J. Biotechnol. *13*(6), 1–7. http://dx.doi.org/10.2225/vol13-issue6-fulltext-3.

Esmaeili, G., Azizi, M., Aroei, H., and Samiei, L. (2016). Micropropagation of *Astragalus adscendens*: A source of gaz-angabin manna in Iran (Persian manna). J. Agric. Sci. Technol. *18*(3), 741–750. http://jast.modares.ac.ir/article-23-1252-en.html.

Gamborg, O.L., Miller, R.A., and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. *50*(1), 151–158. https://doi.org/10.1016/0014-4827(68)90403-5.

Han, M.S., Noh, S.A., Kwak, M.C., and Moon, H.K. (2014). Micropropagation of a rare plant species, *Astragalus membranaceus* Bunge var. *alpinus* N. J. Plant Biotechnol. *41*, 100–106. http://dx.doi.org/10.5010/JPB.2014.41.2.100.

Hartmann, H.T., Kester, D.E., Davies, F.T., and Geneve, R.L. (2011). Hartmann and Kester's Plant Propagation: Principles and Practices (8th edn.) (New Jersey, U.S.A.: Prentice Hall).

Hou, S.W., and Jia, J.F. (2004). High frequency plant regeneration from *Astragalus melilotoides* hypocotyl and stem explants via somatic embryogenesis and organogenesis. Plant Cell Tiss. Organ Cult. *79*(1), 95–100. https://doi.org/10.1023/B:TICU.0000049445.69802.50.

Hrynkiewicz-Sudnik, J., Sĉkowski, B., and Wilczkiewicz, M. (2001). Rozmnażanie Drzew i Krzewów Liściastych (Warszawa: PWN), p. 356–376.

Karagöz, A., Turgut-Kara, N., Çakır, O., Demirgan, R., and Arı, Ş. (2007). Cytotoxic activity of crude extracts from *Astragalus chrysochlorus* (Leguminosae). Biotechnol. Biotechnol. Equip. *21*(2), 220–222. https://doi.org/10.1080/13102818.2007.10817449.

Khanal, N., Schellenberg, M.P., and Coulman, B.E. (2015). Standardizing vegetative propagation as a tool for polycross mating of ascending purple milkvetch (*Astragalus adsurgens* Pall.). College of Agriculture and Bioresources, Soils and Crops Conference Proceedings. http://hdl.handle.net/10388/8857.

Kougioumoutzis, K., Kalpoutzakis, E., and Constantinidis, T. (2017). Multivariate morphometric analysis and taxa delimitation in two narrow Greek endemics: *Astragalus maniaticus* and *Aethionema saxatile* subsp. *corinthiacum*. Plant Biosyst. *151*(1), 108–116. http://dx.doi.org/10.1080/11263504.2015.1103797.

Krigas, N., and Maloupa, E. (2008). The Balkan Botanic Garden of Kroussia, Northern Greece: A garden dedicated to the conservation of native plants of Greece and the Balkans. Sibbaldia *6*, 9–27. https://doi.org/10.23823/Sibbaldia/2008.33.

Lloyd, G.B., and McCown, B.H. (1980). Commercially feasible micropropagation of mountain laurel (*Kalmia latifolia*) by use of shoot-tip culture. Proc. Intl. Plant Prop. Soc. *30*, 421–437.

Luo, J.P., and Jia, J.P. (1998a). Callus induction and plant regeneration from hypocotyl explants of the forage legume *Astragalus adsurgens*. Plant Cell Rep. *17*, 567–570. https://doi.org/10.1007/s002990050443.

Luo, J.P., and Jia, J.P. (1998b). Plant regeneration from callus protoplasts of the forage legume *Astragalus adsurgens* Pall. Plant Cell Rep. *17*(4), 313–317. https://doi.org/10.1007/s002990050399.

Luo, J.P., Jia, J.F., Gu, Y.H., and Liu, J. (1999). High frequency somatic embryogenesis and plant regeneration in callus cultures of *Astragalus adsurgens* Pall. Plant Sci. *143*(1), 93–99. https://doi.org/10.1016/S0168-9452(99)00023-0.

Maloupa, E., Krigas, N., Grigoriadou, K., Lazari, D., and Tsoktouridis, G. (2008). Conservation strategies for native plant species and their sustainable exploitation: Case of the Balkan Botanic Garden of Kroussia, N. Greece. In Floriculture Ornamental Plant Biotechnology: Advances and Topical Issues, Vol. 5, 1st edn., J.A. Teixeira da Silva, ed. (Isleworth, U.K.: Global Science Books), p. 37–56.

Mirici, S. (2004). Endemik geven (*Astragalus polemoniacus* Bunge) bitkisinin yaprak sapı ve yaprak ekspalntlarından yüksek oranda adventif sürgün rejenerasyonu. Selçuk Üniversitesi Ziraat Fakültesi Dergisi *18*(34), 31–34.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. *15*(3), 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

Rios, J.L., and Waterman, P.G. (1997). A review of the pharmacology and toxicology of *Astragalus*. Phytother. Res. *11*(6), 411–418. https://doi.org/10.1002/(SICI)1099-1573(199709)11:6<411::AID-PTR132>3.0.CO;2-6.

Sief-Eldein, A.G.M., El-Arabi, N.I., Sharaf, A.N., AbdAlhady, M.R.A., and Abdallah, N.A. (2018). Regeneration and genetic conservation of the endangered *Astragalus trigonus* plants. Biosci. Res. *15*(1), 19–27.

Tang, W. (1992) Chinese Drugs of Plant Origin (Berlin: Springer Verlag), p. 191–197. https://doi.org/10.1007/978-3-642-73739-8.

Turgut-Kara, N., and Arı, Ş. (2006). Micropropagation of *Astragalus maximus* Willd. Biotechnol. Biotechnol. Equip. *20*(1), 20–22. https://doi.org/10.1080/13102818.2006.10817298.

Turgut-Kara, N., and Arı, Ş. (2008). *In vitro* plant regeneration from embryogenic cell suspension culture of *Astragalus chrysochlorus* (Leguminoseae). Afr. J. Biotechnol. *7*(9), 1250–1255. https://doi.org/10.4314/AJB.V7I9.58657.

Uranbey, S., Cocu, S., Sancak, C., Parmaksiz, I., Khawar, K.M., Mirici, S., and Özcan, S. (2003). Efficient adventitious shoot regeneration in cicer milkvetch. Biotechnol. Biotechnol. Equip. *17*(1), 33–37. https://doi.org/10.1080/13102818.2003.10819192.

Vicente, M.J., Segura, F., Aguado, M., Migliaro, D., Franco, J.A., and Sánchez, J.J. (2011). Genetic diversity of *Astragalus nitidiflorus*, A critically endangered endemic of SE Spain, and implications for its conservation. Biochem. Syst. Ecol. *39*(3), 175–182. https://doi.org/10.1016/j.bse.2011.03.005.

Yorgancilar, M., and Erisen, S. (2011). The effect of thidiazuron (TDZ) on shoot regeneration of *Astragalus schizopterus*. J. Anim. Plant Sci. *21*(3), 519–524.

Received: Sep. 2, 2021
Accepted: Oct. 15, 2021

Address of authors:
K. Grigoriadou*, V. Sarropoulou, N. Krigas and E. Maloupa
Hellenic Agricultural Organization (HAO)-DEMETER, Institute of Plant Breeding and Genetic Resources, Laboratory of Protection and Evaluation of Native and Floriculture Species, Balkan Botanic Garden of Kroussia, P.O. Box 60458, P.C. 570 01 Thermi, Thessaloniki, Greece
* Corresponding author; E-mail: grigokat@outlook.com