Acta Bot. Croat. 77 (1), 70–79, 2018
DOI: 10.1515/botcro-2017-0011

CODEN: ABCRA 25
ISSN 0365-0588
eISSN 1847-8476

Media composition affects seed dormancy, apical dominance and phenolic profile of *Knautia sarajevensis* (Dipsacaceae), Bosnian endemic

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Abstract – *Knautia sarajevensis* is an endemic plant of the Dinaric Alps and is mainly distributed on Bosnian Mountains. Due to the quite large flower heads and easy maintenance, this plant has a potential use as a substitute ornamental plant for *K. arvensis* in perennial beds. The current study evaluated the germination process in different treatments in an attempt to suppress dormancy and increase germination rate, and to develop a successful protocol for micropropagation. An over 60% germination rate was achieved through cultivation of seeds on MS basal medium with reduced mineral nutrient composition and the absence of sucrose. On the other hand, a below 10% germination rate was achieved with untreated seeds. Suppression of apical dominance was achieved through application of high concentrations of kinetin, apical shoot decapitation or cultivation of shoots in liquid media. Overall, liquid cultures were more successful as a micropropagation system for this plant. Shoots spontaneously developed roots on multiplication treatments and were successfully acclimatized. Moreover, phenolic compound profile was analysed in the light of the possible medicinal potential of this plant. Variable amounts of total phenolic compounds as well as individual phenolics were recorded, according to treatment and solidification of media. An increase in rosmarinic acid content was reported for kinetin treatments and acclimatized plants comparing to mother plants in natural habitat. The present study shows that choice of cytokinin concentration, explant type as well as culture type influences not only shoot proliferation and apical dominance suppression but also *in vitro* production of phenolics.

Keywords: apical dominance, acclimatisation, endemic plant, *Knautia sarajevensis*, phenolics, seed dormancy

Introduction

Knautia sarajevensis (Beck) Szabó is an endemic species of the Dinaric Alps that can be found in wood margins and woodland meadows in Bosnia. As an endemic plant species it is included in the Red List of flora for the Federation of Bosnia and Herzegovina and it is mainly found on mountains around Sarajevo (Mt. Igman, Bjelasnica, Trebevic, Jahorina, Ozren; Đug et al. 2013). All of these mountains are subject to great anthropogenic impact due to tourism and to their role as popular recreational sites. These regions are also subject to constant deforestation and neglect, which affects habitat characteristics and plant life, especially plants like *K. sarajevensis*. *Knautia sarajevensis* is a clonal species and its reproduction is achieved through branching of vegetative organs, while seed germination and establishment of fully grown plants is considered to be rare and usually is linked to population establishment at new sites. Poor germination is probably an effect of seed dormancy, elaiosome presence and obligatory light induced germination (Mayer and Svoma 1998). Since *K. arvensis* is a popular ornamental plant used in perennial beds (Hartmann et al. 2010) *K. sarajevensis* too

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must have a potential use as an ornamental and could replace *K. arvensis* due to its larger flower heads and number of flowers than *K. arvensis*. Usually *K. arvensis* is propagated by dormant crown division or stem cuttings.

In vitro culture research into the family Dipsacaceae is very scattered, and only micropropagation protocols for *Scabiosa columbaria* producing an average of 3.0 shoots per explant (Romeijn and Van Lammeren 1999) and *S. caucasica* (Hosoki and Nojima 2004) are available. Apical dominance is a term that signifies apical shoot growth and the inhibition of axillar shoot growth in perennial plants (Cline and Sadeski 2002), and can be a problem in the micropropagation of these species. In its natural habitat *K. sarajevensis* shows apical dominance of flowering over rosette formation and in the first year of growth, formation of a ground rosette takes place, while shoots bearing the flower heads are produced the following year. The reason for this probably lies in the importance of accumulation of a critical mass for flowering, as already recorded for *Succisa pratensis* (Jongejans et al. 2006). Emergence of apical dominance in *in vitro* culture of *K. sarajevensis* is probably the result of a lack of critical mass accumulation and must be supressed in order to produce shoots *in vitro*.

The potential medicinal properties of the *Knautia* genus are still not well explored although there are data about *Knautia arvensis* plants as remedies for various skin disorders, and tea made from flowers and leaves of this plant can be used for many lung problems (Grieve 1931); this species is listed as a relaxant and blood purifier (Mattalia et al. 2013). *Knautia bidens* is listed as a rich source of phenolic compounds comparable to some *Salvia* species (Alali et al. 2007). *Dipsacus* genus has been broadly investigated for its medical properties, and there is evidence about its cytotoxic and anticomplementary activities (Oh et al. 1999; Hung et al. 2005). Since *Dipsacus* and *Knautia* genera are closely related, they could share the same or similar medicinal potentials, so the importance of a micropropagation protocol development lies not only in the need for conservation but also in the potential medicinal uses of the genus *Knautia*. Accordingly, the aim of this study was to develop a successful micropropagation protocol, through plant regeneration from seeds, for conservation purposes. Also production of phenolic compounds was taken under consideration in relation to concentrations of phenolics in mother plants.

Materials and methods

Aseptic seed germination

Fully ripped fruits of *Knautia sarajevensis* were collected from a mother plant from a population located on Mt Igman. A voucher specimen of the seeds was marked and stored in the herbarium of the National Museum of Bosnia and Herzegovina (No. 51413). All fruits were cleaned, elaiosomes were removed as was epicalyx. Seeds were counted and separated into groups of 250 seeds for further treatments (Tab. 1). Prepared seeds were washed with 70% (v/v) ethyl alcohol for 30 sec, followed by 20 min submergence in 20% (v/v) solution of commercial sodium hypochlorite (4% active chlorine), then rinsed five times with sterile-distilled water.

The experiment was conducted through a randomized design with 10 petri dishes containing 20 mL of media, and in each petri dish 25 seeds were inoculated for each treatment. Two sets of ten petri dishes for evaluation of germination rate were used, one set for a 30 day, and other set for a 60 day period. All media were prepared according to Murashige and Skoog (1962) (MS) basal salt composition, pH was adjusted to 5.8 prior to agar addition (0.8%) and were sterilised in an autoclave (1 bar, 121 °C, for 20 minutes). Sucrose (3.0%) was added before pH adjustment to all treatments except KS11 and KS12 (Tab. 1). Gibberellic acid (GA) was filter-sterilised and added after sterilisation of the media for appropriate treatments. All cultures were kept at 23 °C in growth chambers (70% humidity, 2000 lux light intensity, 16 h

Tab. 1. Breaking dormancy and germination rate of *Knautia sarajevensis* seeds. MS – media composition according to Murashige and Skoog (1962), GA – gibberelic acid. Mean values not sharing the same letter(s) within one column are significantly different (P=0.01) according to Newman-Keuls test.

	Pre-treatment	Treatment	Incubation temperature	% germination 30 days	% germination 60 days
KS1	–	MS; 3% sucrose; 0 mg L^{-1} GA	23 °C	9.78±0.01^{h}	24.51±0.00^{i}
KS2	–	MS; 3% sucrose; 0.15 mg L^{-1} GA	23 °C	9.95±0.01^{h}	31.94±0.00^{g}
KS3	30 days at +4 °C	MS; 3% sucrose; 0 mg L^{-1} GA	23 °C	14.59±0.02^{g}	41.15±0.00^{e}
KS4	30 days at +4 °C	MS; 3% sucrose; 0.15 mg L^{-1} GA	23 °C	19.62±0.01^{e}	61.13±0.01^{a}
KS5	30 days at +4 °C	MS; 3% sucrose;0 mg L^{-1} GA	48 h +4 °C; +23 °C	30.15±0.00^{c}	48.55±0.00^{c}
KS6	30 days at +4 °C	MS; 3% sucrose; 0.15 mg L^{-1} GA	48 h +4 °C; +23 °C	33.05±0.01^{b}	60.54±0.00^{b}
KS7	30 days at +4 °C; part of endosperm removed	MS; 3% sucrose; 0 mg L^{-1} GA	23 °C	17.39±0.01^{f}	40.61±0.00^{f}
KS8	30 days at +4 °C; part of endosperm removed	MS; 3% sucrose; 0.15 mg L^{-1} GA	23 °C	19.14±0.00^{e}	48.15±0.00^{d}
KS9	30 days at +4 °C; isolated embryo	MS; 3% sucrose; 0 mg L^{-1} GA	23 °C	2.26±0.00^{j}	3.03±0.01^{l}
KS10	30 days at +4 °C; isolated embryo	MS; 3% sucrose; 0.15 mg L^{-1} GA	23 °C	7.63±0.00^{i}	7.90±0.00^{k}
KS11	–	½ MS; 0% sucrose 0 mg L^{-1} GA	23 °C	27.06±0.02^{d}	48.20±0.00^{j}
KS12	–	½ MS; 0% sucrose 0.15 mg L^{-1} GA	23 °C	65.60±0.01^{a}	90.60±0.01^{h}

photoperiod). Obtained seedlings were further used for the establishment of the *K. sarajevenisis* shoot cultures.

Establishment of the shoot cultures and apical dominance suppression

Roots were removed from all germinated seedlings prior to cultivation on shoot culture media. The obtained shoots were decapitated and apical and nodal parts were separately cultivated on media containing cytokinins (kinetin, 6-benzyladenin and zeatin respectively) and indole-3-butyric acid (IBA). Cytokinins were added in 3 different concentrations, i.e. 0.1, 1.0 and 10.0 mg L^{-1} alone or in combination with 0.1 mg L^{-1} of IBA. Treatments without plant growth regulators (PGR free) were used as control. Each treatment was represented by 5 Erlenmeyer flasks in three independent replications, and in each flask 5 explants were inoculated. All cultures were kept at 23 °C in a growth chamber (70% humidity, 2000 lux light intensity; 16 h photoperiod). After 21 days of cultivation the multiplication rate (number of reactive explants) (MR), multiplication index (average number of produced shoots per explant) (MI), presence of roots and presence of callus (Tab. 2) were recorded.

Evaluation of the media consistency effect on apical dominance suppression

In order to evaluate the media consistency effect on apical dominance suppression and biomass production, shoots about 5 cm in length with minimum of two pairs of leaves were cultivated in solid and liquid media with the addition of 0.1, 1.0 and 10.0 mg L^{-1} of kinetin (KIN). Treatment without PGR was used as control. All media contained 3.0% of sucrose, were prepared according to the MS procedure, and had 0.8% agar (only solid cultures). Media were sterilised in an autoclave, and all cultures were kept at 23 °C in a growth chamber (70% humidity, 2000 lux light intensity; 16 h photoperiod). All treatments were incubated in a shaker (65 rpm), and comprised 5 Erlenmeyer flasks per treatment, with three independent replicates for all treatments. Every Erlenmeyer flask contained 50 mL of media and 5 explants (shoots; 75 per treatment). After 21 days of cultivation for all treatments the multiplication index, and the presence of roots and callus were recorded.

Plantlet acclimatisation

During shoot multiplication, good rooting was achieved and no additional rooting was necessary. Plants with developed roots were washed in tap water to remove the agar and then transferred into pots containing a mixture of soil and sand (3:2). All pots were kept under a 16 h photoperiod (2000 lux light intensity), and constant humidity (70%) and temperature (+23 °C). During the period of 15 days plants were gradually exposed to lower humidity (40%) and larger temperature fluctuation (±10 °C). After 20 days, the development of new leaves was noticed and plants were transferred in greenhouse.

Spectrophotometric analysis of phenolic compounds

Chemical analysis was done for mother plants and regenerated *in vitro* plants as well as acclimatized plants (5 plants per sample). Phenolics were isolated from the aerial parts of plants by maceration in 80% methanol (HPLC grade) by incubation for 24 h at 4 °C. Extracts were centrifuged at 2000 rpm for 15 min, and supernatants were collected for further analysis. Total phenolic content was analysed according to Wolfe et al. (2003) using Folin-Ciocalteu reagent. Quantification was done according to calibration curve of gallic acid and expressed as mg of gallic acid equivalent per g of dry weight (mg GAE/g DW). Total flavonoid content was done using the aluminium chloride method (Ordoñez et al. 2006). Concentration of flavonoids was estimated using the calibration curve of catechin. Results were expressed as mg of catechin equivalent per g of dry weight (mg CE/g DW). Total flavanol content was analysed according to modified method of Gadzovska et al. (2007) using 1% DMACA (w/v) reagent (*p*-dimethyl aminocinnamaldehyde in HCl:CH_3OH, 8:92) and using calibration curve of catechin. Results were expressed as mg of catechin equivalent per g of dry weight (mg CE/g DW). Total proanthocyanidins content was analysed using vanillin-HCl method (Wettstein et al. 1977) and

Tab. 2. Effects of decapitation and kinetin application on the multiplication rate and index of *Knautia sarajevensis* cultivated in solid media. MR – multiplication rate (percentage of explants with formatted shoots), MI – multiplication index (average number of formatted shoots per explant), KIN – kinetin, IBA – indole-butyric acid. Mean values not sharing the same letter(s) within one column are significantly different (P=0.01) according to Newman-Keuls test.

KIN (mg L^{-1})	IBA (mg L^{-1})	Nodal explants				Apical explants			
		MR	MI	Roots	Callus	MR	MI	Roots	Callus
0.0	0.0	45.15	3.10±0.10^{c}	–	–	0.00	1.00±0.00^{b}	–	–
0.1	0.0	52.13	5.08±0.35^{a}	good, long	–	0.00	1.00±0.00^{b}	small, under-developed	–
0.1	0.1	43.15	2.80±0.33^{c}	good, long	–	0.00	1.00±0.00^{b}	short	–
1.0	0.0	42.34	2.40±0.07^{c}	short, thick	–	0.00	1.00±0.00^{b}	short	small, with indirect root regeneration
1.0	0.1	32.15	2.05±0.01^{c}	longer	–	0.00	1.00±0.00^{b}	short	
10.0	0.0	45.56	3.70±0.08^{b}	–	–	1.00	2.00±0.05^{a}	–	–
10.0	0.1	44.43	3.30±0.01^{c}	–	–	0.00	1.00±0.00^{b}	–	with shoot regeneration

quantified according to the calibration curve of catechin. Results were expressed as mg of catechin equivalent per g of dry weight (mg CE/g DW). Samples with the highest total phenolic content were further analysed by HPLC-UV technique.

HPLC analysis of phenolic compounds

Separation and analysis of flavonoids and phenolic acids (hydroxycinnamic and hydroxybenzoic acids) were performed on a Shimadzu LC-2010c HPLC using Phenomenex Kinetex C18 (2.6 μm ID, 150 × 4.6 mm) column. The mobile phase included component A (20 mM formic acid in water) and component B (acetonitrile) in a defined gradient (0 min 5% B; 4 min 5% B; 54 min 40% B; 60 min 40% B; 60, 5 min 5% B; 70 min 5% B; 70 min stop) at flow rate 0.4 mL/min. HPLC was equipped with UV detector, and absorbance was monitored at 270 nm. Concentration of flavonoid components was calculated in relation to calibration curves of standards: myricetin, quercetin, naringenin, apigenin, kaempherol, chrisin, pinocembrin and galangin. For phenolic acids standards of chlorogenic, caffeic, sinapic, ferulic, rosmarinic, gallic, 4-hydroxybenzoic, vanillic, syringic and salicylic acids were used (concentration range: 1×10^{-7} mol L^{-1} – 1×10^{-3} mol L^{-1}). All standards used were purchased from Sigma and were of at least analytical grade.

Statistical analysis

All results were expressed as the mean values (±standard deviation; STDEV) of the three independent replicates. Analysis of variance of parametric data was done according to ANOVA test using Newman-Keuls test as a post hoc analysis. Differences between treatments were evaluated at $p<0.01$. Correlation coefficient was calculated according to Pearson product-moment correlation coefficient at $p<0.05$. All statistical testing was done using Statistica 10.0 software (Copyright© StatSoft. Inc. 1984–2011).

Results

Aseptic seed germination

Seeds of *K. sarajevensis* have elaiosomes and the removal of elaiosomes and seed coat was necessary for dormancy suppression. Isolation of embryos, one of the methods for dormancy suppression, was also conducted. Different cold pre-treatments for elaiosome-free seeds were used for improvement of the germination rate in combination with gibberellic acid application. The efficiency of gibberellin stimulation of seed germination in *K. sarajevensis* depended upon the pre-treatment and cultivation conditions. Seedlings developed a pair of leaves 15 days after cultivation but germination time was unsynchronized within one treatment. When no cold pre-treatment was applied maximum germination rate was prolonged up to 2 months even with GA application. Incubation of non-imbibed seeds at +4 °C for 30 days increased germination percentage. Further incubation at 4 °C after imbibition of cold pre-treated seeds (KS5 and KS6 treatments) significantly increased seed germination especially in combination with GA application and maximum germination rate was achieved in the first 30 days (Tab. 1). Removal of sucrose in combination with reduction of basal salts in the media significantly induced germination and reduced duration of germination period, especially when GA was applied (Tab. 1), moreover up to 65% of the seeds germinated in first 30 days.

Establishment of the shoot cultures and apical dominance suppression

In preliminary research (data not shown), seedlings were cultivated on media containing 0.1, 1.0 and 10.0 mg L^{-1} BA (6-benzyladenine), ZEA (zeatin) and kinetin (KIN) in solid media. During cultivation, emergence of apical dominance was noticed and no shoot formation was recorded. Shoots cultivated on solid media containing BA and ZEA showed signs of chlorosis and root formation was very low, while kinetin induced good root formation, and no chlorosis was noted but apical dominance was present. For further multiplication and apical dominance suppression seedlings from which apical and nodal explants were derived were used. Apical parts of the shoots and nodal segments were separately cultivated on media containing different concentrations of kinetin alone or in combination with 0.1 mg L^{-1} IBA. Cultivation of explants in kinetin-containing media provided different responses depending upon kinetin concentration and explant type. Apical explants did not produce any shoots except when the highest concentration of KIN was applied (10 mg L^{-1}), while nodal explants produced shoots in different frequencies. The highest multiplication rate and index were reported for treatment with 0.1 mg L^{-1} of KIN using nodal explants (Tab. 2, Fig. 1). High concentrations of KIN in this study also induced callus formation in the basis of nodal explants with indirect shoot regeneration (Tab. 2, Fig. 1). Addition of IBA to the media had inhibitory effects on shoot and root formation (Tab. 2, Fig. 2). Plants with good roots were acclimatized in greenhouse conditions with a survival rate over 70%. All plants formed good ground rosettes comprising up to 10 leaves with no shoot formation demonstrating a 2 year vegetation period as recorded in the natural habitat.

Evaluation of liquid media effect on apical dominance suppression

In solid culture shoots were short (5–7 cm) and no elongation was noticed and apical dominance suppression was only possible by removal of shoot apex or by application of high kinetin concentrations. In contrast, the highest concentration of KIN (10 mg L^{-1}) was unfavourable in liquid system and plants decayed within 3 days and this treatment was not further analysed. Shoots cultivated in the liquid media containing 0.1 mg L^{-1} of KIN demonstrated a 100% multiplication rate and multiplication was achieved mainly through proliferation of several axillary buds (Fig. 3A). Also, development of elongated shoots was reported (Fig. 3B). Apical dominance was suppressed and production of up to 5.4 shoots per explant was achieved using a low concentration of kinetin.

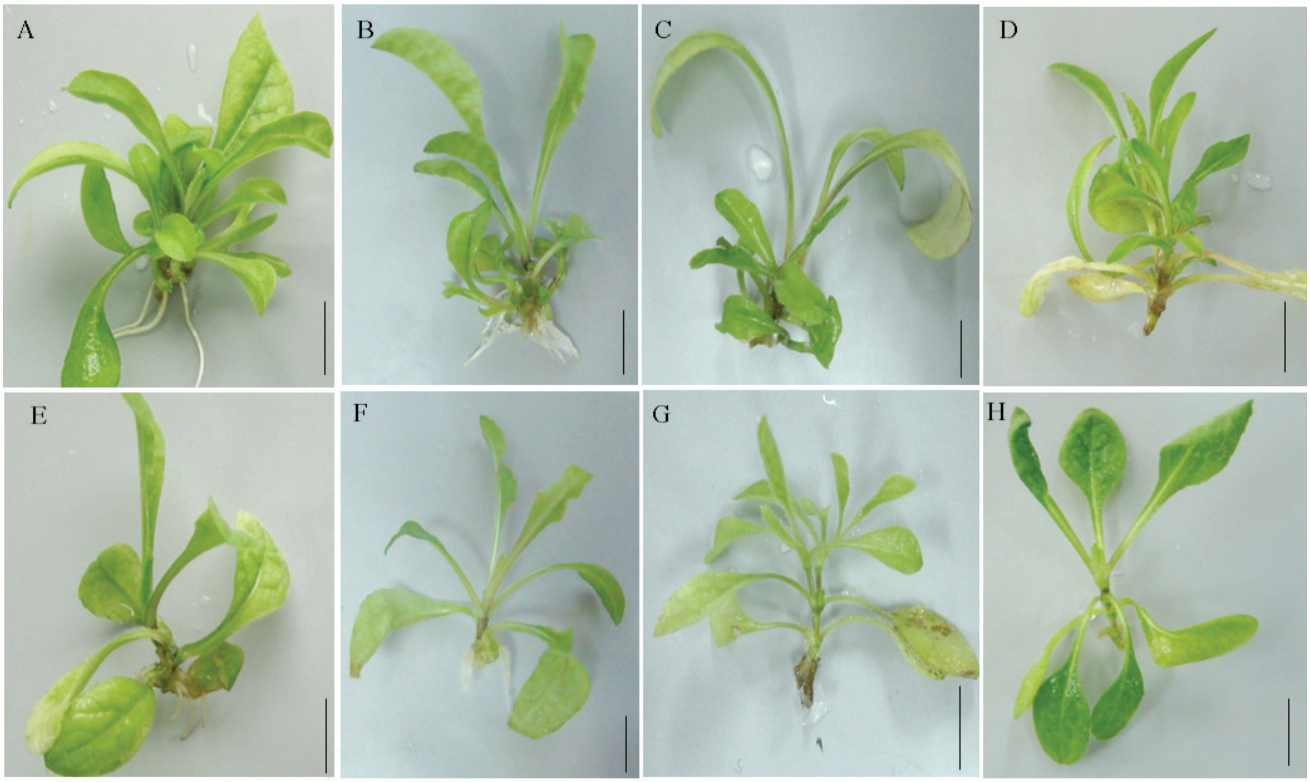

Fig. 1. Effects of decapitation and kinetin application on apical dominance suppression in *Knautia sarajevensis*; nodal explants cultivated in solid media containing kinetin: (A) 0.1 mg L^{-1}, (B) 1 mg L^{-1}, (C) 10 mg L^{-1}, D) 0 mg L^{-1}; apical explants cultivated in media containing kinetin: (E) 0.1 mg L^{-1}, (F) 1 mg L^{-1}, (G) 10 mg L^{-1}, (H) 0 mg L^{-1}. Bars = 1 cm.

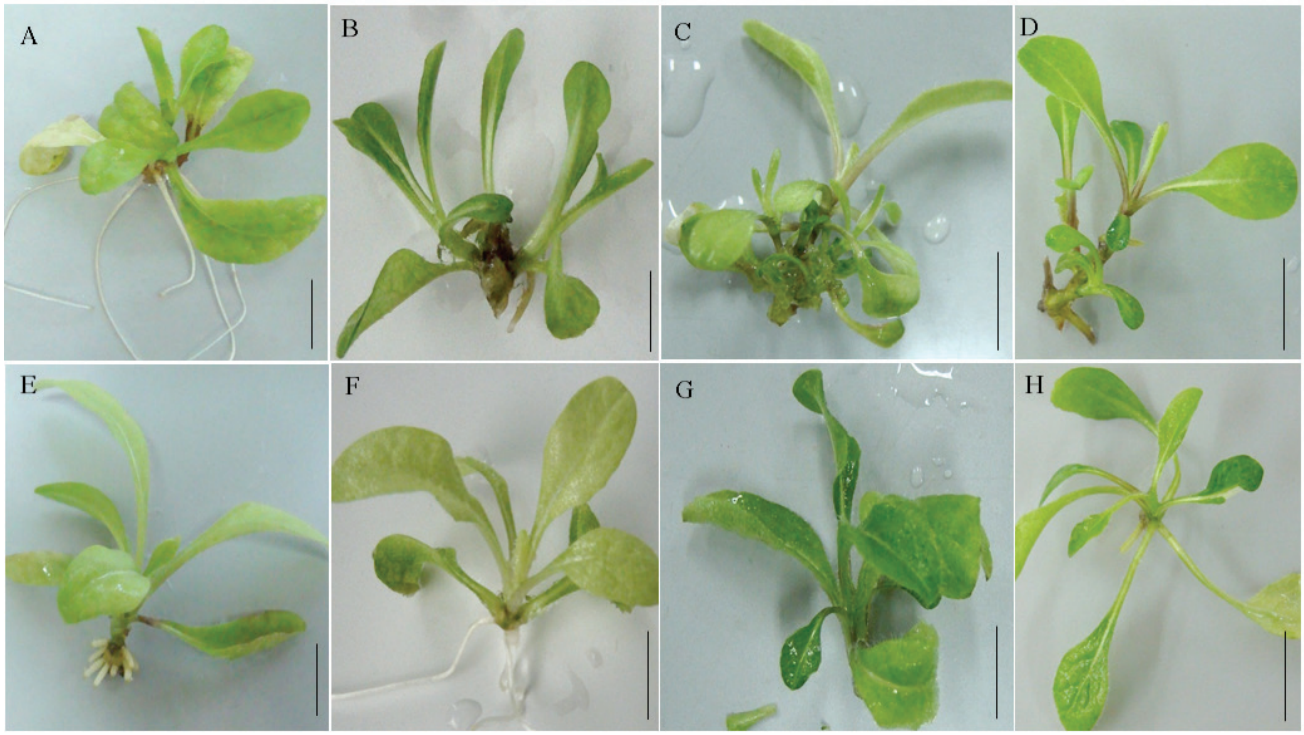

Fig. 2. Effects of decapitation and kinetin (KIN) application in combination with indole-3-butyric acid (IBA) on apical dominance suppression in *Knautia sarajevensis*; nodal explants cultivated in solid media containing: (A) 0.1 mg L^{-1} KIN and 0.1 mg L^{-1} IBA, (B) 1 mg L^{-1} KIN and 0.1 mg L^{-1} IBA, (C) 10 mg L^{-1} KIN and 0.1 mg L^{-1} IBA, (D) 0 mg L^{-1} KIN and 0 mg L^{-1} IBA; apical explants cultivated in solid media containing: (E) 0.1 mg L^{-1} KIN and 0.1 mg L^{-1} IBA, (F) 1 mg L^{-1} KIN and 0.1 mg L^{-1} IBA, (G) 10 mg L^{-1} KIN and 0.1 mg L^{-1} IBA, (H) 0 mg L^{-1} KIN and 0 mg L^{-1} IBA. Bars = 1 cm.

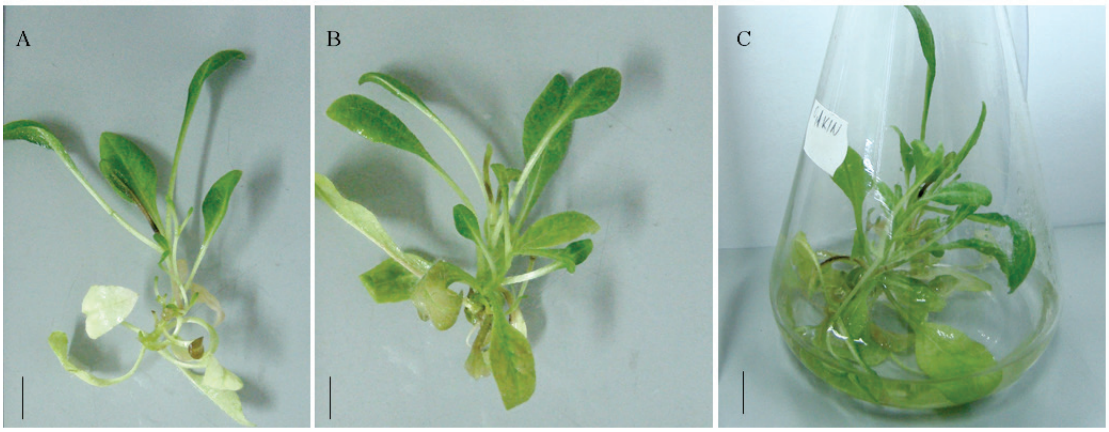

Fig. 3. Apical dominance suppression and multiplication of *Knautia sarajevensis* shoots in liquid culture: A) bud clusters, B) shoot multiplication, and C) liquid media cultivation. Bars = 1 cm.

Higher concentrations of KIN (1 mg L^{-1} and 10 mg L^{-1}) had negative effects on shoot proliferation and induced vitrification of the plants. In this study the use of a small amount of the medium (50 mL) and a low kinetin concentration were enough for the avoidance of vitrification during multiplication (Fig. 3C). Moreover, developing shoots were not submerged in the media because explants floated due to the large leaves and the hairs on the leaves in which air can be trapped.

Phenolic compounds profile

Analysis of total phenolic content in *K. sarajevensis* showed a high concentration of phenols and flavonoids in the aerial part of mother plants (Tab. 3). HPLC analysis showed that around 30% of phenolic profile is composed of phenolic acids (gallic, 4-hydroxybenzoic, vanillic, salicylic, chlorogenic, caffeic, sinapic, ferulic and rosmarinic acids), (Tab. 4) and some flavonoids: myricetin as a most abundant (2.72 nmol mg^{-1}), and apigenin and kaempherol in traces. Similar profile with lower concentration of individual components was found in *in vitro* cultivated plants as well as in acclimatized plants. Elevation of rosmarinic acid concentration was reported for acclimatised plants and some KIN treatments (Tab. 4); in addition, the synthesis of syringic acid under KIN treatment was noticed.

Discussion

Natural populations of endemic *Knautia sarajevensis* are subject to anthropogenic impacts and it is important to develop a micropropagation protocol in order to give opportunities for the growth of this species in new habitats. Micropropagation of endangered species enables plant propagation regardless of the season from a small number of mother plants (Mikulík 1999). For conservation purposes it is recommended to use seed for establishment of the cultures as they have a broader genetic base (Chua and Henshaw 1999).

Removal of elaiosomes alone was not sufficient for dormancy removal in *K. sarajevensis* seeds, and different cold pre-treatments combined with gibberellic acid application were used for improvement of the germination rate. Gibberellic acid alone or in combination with cold treatment can break dormancy and promote germination as recorded for

Tab. 3. Phenolic contents in *Knautia sarajevensis* shoots after cultivation in solid and liquid media. K – kinetin, 01 – 0.1 mg L^{-1}, 1 – 1.0 mg L^{-1}, S – solid culture, L – liquid culture, PGR– no plant growth regulators added, MP – mother plant, AP – acclimatised plant. Mean values not sharing the same letter(s) within one column are significantly different (P=0.01) according to Newman-Keuls test.

Treatment	Phenolics (mg g^{-1} DW^{-1})			
	Total phenols	Total flavonoids	Flavanols	Proanthocyanidins
MP	121.04±8.97^{a}	154.51±16.11^{a}	6.19±0.03^{a}	33.46±3.86^{a}
AP	33.16±8.00^{c}	165.13±4.38^{a}	0.94±0.00^{cb}	21.50±0.77^{b}
PGR S	31.14±3.68^{cd}	63.27±11.71^{b}	0.83±0.12^{cd}	2.09±0.04^{e}
PGR L	21.98±0.15^{e}	27.86±1.70^{cd}	0.53±0.11^{cd}	14.63±0.07^{c}
01K S	20.31±3.55^{e}	25.57±2.48^{cd}	0.31±0.01^{d}	3.46±0.04^{e}
01K L	24.488±3.62^{de}	34.24±4.84^{cd}	0.80±0.01^{cd}	9.40±0.42^{d}
1K S	26.02±2.52^{d}	33.88±3.18^{cd}	0.46±0.02^{cd}	3.73±0.02^{e}
1K L	56.50±2.67^{b}	49.24±4.02^{bc}	1.51±0.03^{b}	4.16±0.46^{e}

Tab. 4. HPLC analysis of phenolic compounds in *Knautia sarajevensis* shoots cultivated in solid and liquid media. K – kinetin, 1 – 1.0 mg L^{-1}, S – solid culture, L – liquid culture, PGR– no plant growth regulators added, MP – mother plant, AP – acclimatised plant, nd – not detected.

Hydroxybenzoic acid (nmol mg^{-1} of extract)	Treatment					
	MP	AP	PGR S	PGR L	1K S	1K L
Gallic acid	8.71	4.75	2.88	1.23	6.15	nd
4-Hydroxybenzoic acid	46.05	11.07	14.40	7.16	16.41	2.72
Vanillic acid	0.38	0.62	0.12	0.37	4.87	1.35
Syringic acid	nd	0.36	nd	0.19	3.09	0.94
Salicylic acid	20.59	11.40	12.75	7.19	18.97	4.18
Chlorogenic acid	1.67	0.18	1.30	0.43	0.32	2.49
Caffeic acid	0.031	0.59	0.32	0.35	0.20	nd
Sinapic acid	53.65	1.98	0.61	0.63	0.95	0.10
Ferulic acid	57.69	2.59	0.56	nd	0.94	0.27
Rosmarinic acid	2.61	12.71	13.86	5.01	11.66	2.61
Myricetin	2.72	0.47	nd	0.80	0.77	nd
SUM	194.11	45.89	46.81	23.39	64.27	14.66

many different genera (Nau 1996, Raeber and Lee 1991). Efficiency of gibberellin stimulation of seed germination in *K. sarajevensis* depended upon the pre-treatment and cultivation conditions used, but was usually pronounced when imbibed seeds were additionally exposed to cold treatment as previously recorded for other species (Yamauchi et al. 2004, Okamoto et al. 2006). Endosperm plays an important role in seed germination (Yan et al. 2014), synthesis of hydrolytic enzymes, sensing the red and far-red light necessary for germination (Lee et al. 2012) and controlling embryo growth (Lee et al. 2010). Removal of part of the endosperm or isolation of embryos can be a useful tool for seed dormancy removal (Yan et al. 2012) since seed dormancy can be caused through an impermeable seed coat or other dormancy factors from the endosperm (Nonogaki, 2014). In this study such treatments did not stimulate seed germination in *K. sarajevensis* even when GA was applied, as compared to other treatments. An explanation for this could lie in the composition of *K. sarajevensis* endosperm. Seeds of the *Knautia* genus contain oils as a reserve of nutrients (Mayer and Svoma 1998, Tonguç and Erbaş 2012). For germination and energy gain, oils from the endosperm must be catabolised though gluconeogenesis in order to be passed to the embryo in the form of sucrose (Penfield et al. 2004). Endosperm removal excludes the reserve oils in which case embryos are under stress since they have no energy source. It is recorded that embryos in suboptimal growth conditions experience restriction of the gluconeogenic pathway (Rylott et al. 2003), which could lead to reduction in the growth and germination rate. Removal of sucrose in combination with a reduction of basal salts in the media was the most successful treatment for elaiosome-free seeds of *K. sarajevensis*. Presence of sucrose in media can induce osmotic stress and inhibit seed germination. Also, growth of the seedlings can be inhibited by sucrose hydrolytic products, which are formed during media sterilisation process (Sawyer and Hsiao 1992, Pan and van Staden 1999). Elevation of germination rate by reduction of media mineral composition is recorded also for other species as well (Mishra et al. 2013).

Apical dominance of ground rosette over flowering stems is present in natural habitats of *K. sarajevensis,* and this phenomenon has previously been recorded for *Succisa pratensis* (Jongejans et al. 2006), and it is probably the reason for the recorded apical dominance emergence in shoot cultures in this study. Choice of an adequate cytokinin and a concentration that stimulates shoot induction and elongation varies depending upon plant species and must be optimised for each particular plant species (Jana et al. 2013). Successful multiplication was achieved by low kinetin concentrations and such a treatment has been recorded for a relatively small number of species (Deo et al. 2014). Elevation of kinetin concentration induced callus formation, which has been reported in *Scrophularia takesimensis* cultures (Ding and Chen 2007), with the possibility for regeneration from petiole and leaf (Wang et al. 2013); similar results were also reported for other *Scabiosa* species (Hosoki and Nojima 2004). Decapitation was relatively successful as a tool for apical dominance suppression in *K. sarajevensis* shoot cultures. Apical shoot removal was previously recorded as an effective method for apical dominance suppression (Podwyszynska 1997). Also it is considered that the endogenous auxin to cytokinin ratio suppresses bud outgrowth and an exogenous supply of cytokinins can change this ratio (Hillman 1984), which in turn stimulates bud outgrowth. Senescence of older leaves was observed for all apical explants and control treatment (no plant growth regulators added), which probably impaired successful multiplication (Mensual-Sodi et al. 2007). Kinetin application reduced senescence in nodal explants, and the role of kinetin in senescence has been previously demonstrated (Mukharjee and Kumar 2007). Negative effects of exogenous auxins on multiplication have been previously reported. Single shoot development and callus induction was demonstrated due to apical dominance resulting from increased auxin concentration (Buah et al. 2010). In this study

high endogenous auxin levels were responsible for root formation on media containing only kinetin while additional exogenous auxins had a negative effect on rhizogenesis. The negative effect of high auxin concentration on root formation has been well documented with reference to a number of species (Buah et al. 2010, North et al. 2010). High survival rate during acclimatisation has been reported for some *Scabiosa* species (Hosoki and Nojima 2004, Wang et al. 2013), but data regarding *Knautia* species are scattered.

Since suppression of apical dominance is possible through the use of liquid cultures (Mehrotra et al. 2007), in the next step we used a liquid culture using the same kinetin concentrations that were favourable for multiplication in a solid culture with no signs of callus formation to ensure that only shoots by direct regeneration were obtained. Development of bud clusters and mechanical separation of shoots in liquid systems provide an efficient delivery system in micropropagation (Levin et al. 1997, Ziv et al. 1998). Production of buds enables the production of a large number of plants (Takayama and Misawa 1981). The contact of the explants with the medium facilitates the uptake of nutrients and plant growth regulators, which lead to promotion of shoot and root formation (Sandal et al. 2001). Forced aeration, due to continuous shaking of the medium, provides oxygen supply to the whole explant, which stimulates growth (Mehrotra et al. 2007). There are many studies suggesting that agar elimination causes vitrification of tissue during micropropagation (John 1986, Kevers et al. 1987). The problem of asphyxiation is a common problem in liquid cultures (Mehrotra et al. 2007). In this study reduction of the media amount and low kinetin concentration were enough for avoidance of vitrification during multiplication. Moreover, developing shoots were not submerged in the media because explants tended to float due to their large leaves and the hairs on the leaves among which air can be trapped. The use of surface tension and floating properties of an explant is very useful for the avoidance of submergence of tissues and vitrification phenomena in a liquid medium (Debergh et al. 1992).

Since closely related members of the Dipsacaceae family have been demonstrated to have medicinal properteis (Oh et al. 1999, Hung et al. 2005, Mattalia et al. 2013), prospects for the micropropagation of *K. sarajevensis* as a potential medicinal plant also included phytochemical screening as the first step towards such a use of the plant. There are no available data about the phenolic compounds found in *K. sarajevensis*, and also very little is known about the genus *Knautia*, in general; some of the identified components are reported for the first time. According to the available data apigenin, swertijaponin, and giganteoside A were found in *K. montana* (Movsumov et al. 2011); cryptochlorogenic and chlorogenic acid and isovitexin 7-β-D-glucopyranoside were detected in *K. arvensis* (Moldoch et al. 2011), while a high concentration of polyphenolics in *K. bidens* has been reported and the plant is considered a rich source of phenolic compounds (Alali et al. 2007). Kinetin is considered to be a promoter of secondary metabolite synthesis (Klessig and Malamy 1994, Kim et al. 2009), as demonstrated in this study. There is less synthesis than in the mother plants, but some components that are not recorded for mother plants have been identified in kinetin-treated *in vitro* plants, which is in accordance with other studies (Rao and Ravishankar 2002, Luczkiewicz and Gold 2005). Manipulation of PGR concentration and culture conditions can provide changes in secondary metabolites (Rao and Ravishankar 2002, Luczkiewicz and Gold 2005, Lucchesini et al. 2009) as also shown in this study. Use of shoots instead of callus is more convenient and it is also considered that more differentiated tissues produce more metabolites (Luczkiewicz and Gold 2005, Nath and Buragohain 2005, Sood and Chauhan 2010), and use of liquid systems gives better results (Savio et al. 2011) as demonstrated in this study.

To conclude, this study provides a successful micropropagation protocol for *Knautia sarajevensis* and accordingly a useful tool in the establishment of new population sites as a method for conservation of this endemic species and for its potential use as an ornamental plant, replacing *K. arvenisis* in perennial beds. Successful micropropagation can be performed by germination of seeds on media without sucrose and reduced basal salt concentration. Apical dominance can be overcome by decapitation of shoots or cultivation in liquid media. Due to the high endogenous concentration of auxin, rooting media is not necessary and roots develop in multiplication media with successful acclimatization of rooted plants. Elevation of secondary metabolite concentration (compared to control) due to kinetin application in solid cultures gives an input for the possible elicitation of phenolic acids in this plant. The presence of phenolic compounds in mother plants as well as *in vitro*-produced plants raises the possibility of the potential medicinal use of this plant.

Acknowledgments

This work was supported, in part, by grant No. LO1204 (Sustainable development of research in the Centre of Region Haná) from the National Program of Sustainability I, Ministry of Education, Youth and Sports, Czech Republic. We thank Karel Doležal for stimulating discussions and a critical reading of the manuscript.

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