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Effect of Medium Salt Strength on the Micropropagation, Phenolic Content and Antioxidant Activity of *Arnica montana* L., Threatened Plant Species

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ABSTRACT: The effect of different strength of Murashige and Skoog (MS) medium (full, half-, third and quarter content of salts and vitamins) on micropropagation, shoot growth, in vitro rooting and antioxidant properties of *Arnica montana* was demonstrated. The full strength MS medium supplemented with 1 mg L^{-1} 6-benzylaminopurine (BAP) and 0.1 mg L^{-1} indole-3-acetic acid (IAA) was the best for plant multiplication (11.4 number of shoots per explant) and biomass production (FW 1.66 g and DW 0.25 g). Optimal rooting medium was half strength MS rooting medium contained 0.5 mg L^{-1} indole-3-butyric acid (IBA), although the maximum number of roots was induced on quarter strength MS rooting medium. The rooted plants were successfully ex vitro acclimatized. The highest phenolic content and antioxidant activity were recorded in shoots grown on ¼ strength of MS medium, whether derived from micropropagation or from rooting stage. This study showed that tissue culture of *A. montana* is alternative source of natural antioxidants.

Keywords: *A. montana*; in vitro culture; shoot growth; phenols; antioxidants.

INTRODUCTION

Arnica montana L. is a valuable medicinal plant spread in various regions of Europe (Lange, 1998). It is a rare and endangered species according to International Union for Conservation of Nature (Bilz *et al*., 2011). The plant is rich in sesquiterpene lactones, flavonoids, phenolic acid, essential oils etc. (Willuhn, 1998; Merfort, 2007). *A. montana* has a long history of use in the folk medicine, and presently is largely applied in pharmacy and cosmetics due to its antiseptic, anti-inflammatory, antibacterial, antifungal and antioxidant activities (Shiffman *et al.*, 2012). Development of biotechnological methods is of a great importance for multiplication and conservation of this endangered species and for enhancement of biologically active compounds particularly those with antioxidant properties. For micropropagation, most widely used culture medium is Murashige and Skoog, 1962 (MS) medium, because most of the plants respond favorably

to this medium, contained all the nutrients essential for in vitro plant growth (Kumar and Reddy, 2011). Full strength of salts in media provide good results for numerous species, but in some plants and for specific purposes the reduction of salts level to half or quarter of the full concentration gave better results in in vitro growth (Saad and Elshahed, 2012). Most of the authors studied in vitro cultures of *A. montana* recommended using half strength MS medium for rhizogenesis (Conchou *et al.*, 1992; Le, 2000; Petrova *et al*., 2011), however the effect of strength of the MS medium on micropropagation and rooting of the species is not sufficiently

studied. Plant tissue cultures represent a potential source for obtaining biologically active compounds including phenols and antioxidants under strictly controlled conditions. Experimental in vitro system allows studying the influence on the secondary metabolism of a broad spectrum of effects of chemical (change of nutrients contents in culture medium, supplement of plant growth regulators etc) or physical (UV radiation, temperature, etc) nature (Tisserat and Vaughn, 2004). The literature survey showed that there is no information about the antioxidant activity of in vitro cultured *A. montana* plant material depending on the strength of nutrient medium. The objective of the present study is to evaluate the effect of MS medium salt strength on shoot micropropagation, biomass accumulation, rooting efficiency and antioxidant activity of *A. montana*.

MATERIALS AND METHODS

Biotechnological methods

A. Initial plant material

Seeds of *A. montana* (Botanical garden, Chemnitz, Germany) were used to initiate *in vitro* culture. Surface sterilization of mature seeds was performed using 70% (v/v) ethanol for 1 min and commercial bleach ACE for 10 min followed by washing in sterile distilled water (three times). Shoots (1-1.5 cm) each carrying a node were isolated from three-month old micropropagated *A. montana* plants and used as explants for all experiments.

B. Nutrient medium and culture conditions

For shoot multiplication, the nodal segments were cultured on MS nutrient medium containing of either full, half (1/2), third (1/3), and quarter (1/4) strength MS micro-, macro- nutrients and vitamins. All tested nutrient media were supplemented with 1 mg L^{-1} BAP and 0.1 mg L^{-1} IAA. Sucrose and agar concentrations were constant – 30 g L^{-1} and 6 g L^{-1}, respectively. The composition of used medium for micropropagation was as follows:

MSP1 (Full strength MS) with 1 mg L^{-1} BAP and 0.1 mg L^{-1} IAA

MSP2 (1/2 strength of MS) with 1 mg L^{-1} BAP and 0.1 mg L^{-1} IAA

MSP3 (1/3 strength of MS) with 1 mg L^{-1} BAP and 0.1 mg L^{-1} IAA

MSP4 (1/4 strength of MS) with 1 mg L^{-1} BAP and 0.1 mg L^{-1} IAA

After the four week culture period, the average number of shoots induced per explants and the mean height of shoots were recorded. Then, micro propagated shoots (20 single shoots) were collected and washed with distilled water. They were dried on filter paper to remove water and the fresh weight was determined. For dry weight, the shoots were oven dried at 60 °C for 3 days until completely dry and then measured.

In vitro rooting of shoots was carried out using the same MS strength medium (full, ½, 1/3 and ¼ MS) containing 0.5 mg L^{-1} IBA, 20 g L^{-1} sucrose and 6 g L^{-1} agar. The composition of used medium for rooting was as follows:

MSR1 (Full strength MS salts and vitamins) with 0.5 mg L^{-1} IBA

MSR2 (1/2 strength of MS salts and vitamins) with 0.5 mg L^{-1} IBA

MSR3 (1/3 strength of MS salts and vitamins) with 0.5 mg L^{-1} IBA

MSR4 (1/4 strength of MS salts and vitamins) with 0.5 mg L^{-1} IBA

The plant height, the mean number of roots per plant and the mean root length were measured after four weeks culture.

For acclimatization under ex vitro conditions, the rooted plants were carefully taken out from the culture vessels and washed under running tap water to remove the gelling agent. They were transferred to small plastic pots (8 cm diameter) containing mixture: peat: perlite: coconut fiber (2:1:1 v/v/v). The potted plants were covered with a transparent polythene membrane to ensure high humidity (90%) and were opened after two weeks. The percentage of surviving plants was determined after five weeks. After two months, the potted plants were transferred in glasshouse for further acclimatization.

C. Culture conditions

The medium pH was adjusted to 5.7 using 0.1N NaOH or 0.1N HCl before autoclaving at 1 atm (120°C for 20 min). The in vitro cultures were maintained in a growth room at 22±2°C under 16 h photoperiod with light intensity of 40 µM $m^{-2}s^{-1}$ provided by Philips 36 W cool white fluorescent tubes. Ex vitro plants were maintained in a growth chamber at 25±1°C under 16 h photoperiod and 50 µM m^{-2} s^{-1} light intensity.

Biochemical analysis

A. Plant material and extraction procedure

Shoots of *A. montana* derived from micropropagated stage and such from rooting stage were used for determination of total phenol content and antioxidant activity. Air-dried, ground plant material (1 g) was extracted with 80% (3 x 30 mL) methanol by classical maceration for 24 h. After evaporation of the solvent the crude extract was subject to subsequent analysis.

B. *DPPH radical scavenging activity*

The effect of methanolic extracts on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals was estimated according to Stanojević *et al*. (2009). The IC50

values were calculated by Software Prizm 3.00. All of the experiments were carried out in triplicate.

C. Determination of total phenolic content

Total phenolic content of the methanol extracts was determined by employing the method given in the literature involving Folin–Ciocalteu reagent and gallic acid as standard (Giorgi *et al*., 2009; Nićiforović *et al*., 2010). The content of total phenols was presented as mean ± standard deviation of tree independent analyzes (n=3).

D. Statistical analysis of the data

Twenty explants were used for each treatment and the experiment was repeated twice. Data were subjected to one-way ANOVA analysis of variance for comparison of means using a statistical software package (Statigraphics Plus, version 5.1 for Windows). Data were reported as means ± standard error.

RESULTS AND DISCUSSION

A. Effect of medium salt strength on shoot micropropagation

Our preliminary observations showed that MS medium supplemented with 1 mg L^{-1} BAP and 0.1 mg L^{-1} IAA gave the best micropropagation of *A. montana* (Petrova *et al*., 2011). Therefore, these growth regulators were added to studied nutrient media containing different concentrations of salts and vitamins. The strength of medium influenced significantly on the formation of adventitious shoots of *A. montana* (Table 1). The micropropagation was the most effective on medium MSP1 containing full strength of macro, micro- salts and vitamins, 1 mg L^{-1} BAP and 0.1 mg L^{-1} IAA, when the mean number of shoots per explant reached 11.4 after four weeks of culture. A gradual decrease of propagation rate was observed with reducing the salts and vitamins concentrations in culture medium (Fig. 1a).

Table 1. Effect of MS medium salt strength on the micropropagation of *A. montana* after four weeks of culture.

MS medium 1 mg L^{-1} BAP + 0.1 mg L^{-1} IAA	Number of shoots per explant	Height of shoots, cm	Fresh weight, g	Dry weight, g
Full strength (MSP1)	11.40±0.80^{d}	1.79±0.18^{b}	1.66±0.19^{c}	0.25±0.07^{d}
½ (MSP2)	5.25±0.63^{c}	1.58±0.12^{ab}	1.45±0.17^{dc}	0.19±0.08^{ab}
1/3 (MSP3)	4.15±0.32^{b}	1.33±0.14^{a}	1.23±0.14^{ab}	0.16±0.06^{ab}
¼ (MSP4)	2.60±0.18^{a}	1.36±0.11^{a}	1.04±0.11^{a}	0.12±0.04^{a}
LSD	1.01	0.26	0.29	0.12

The data are presented as means of 20 shoots per treatment ± standard error. Different letters indicate significant differences assessed by Fisher LSD test (P ≤ 0.05) after performing ANOVA multifactor analysis.

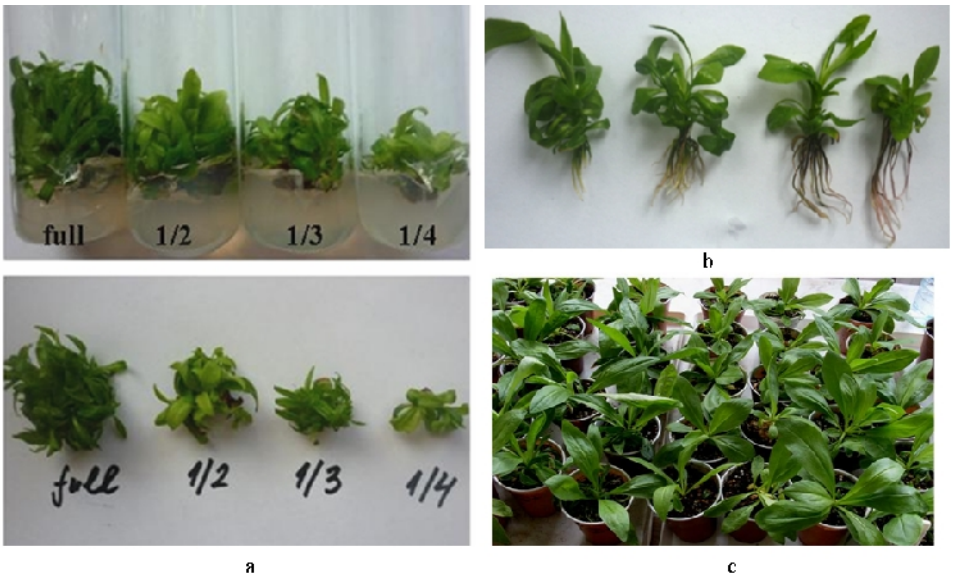

Fig.1. (a). Micropropageted shoots of *A. montana* cultured on different strength MS media (b). *A. montana* plants grown on different strength MS rooting media (c). *Ex vitro* acclimatization of micropropagated plants on mix (peat: perlite: coconut fiber; 2:1:1).

The MSP4 medium contained ¼ of MS salts and vitamins, 1 mg L^{-1} BAP and 0.1 mg L^{-1} IAA was the less effective for micropropagation and only 2.6 shoots per explants were formed. The height of shoots did not differ significantly among the tested nutrient media. However the shoots grown on full strength MSP1 medium reached the greatest height (1.79 cm). The media with low salt and vitamins content reduced propagation efficacy. The MS medium salt level plays an important role on the in vitro propagation potential of *A. montana*. The concentrations of macronutrients, micronutrients and vitamins in the culture media are essential requirements required for cell and tissue growth of most plant species. The effects of MS medium salt strengths were investigated for shoot formation. A significant difference in the number of newly formed shoots was observed among the different strength of MS medium tested. Maximum shoot number per explant was recorded on full strength medium, which was significantly higher than other medium strengths (Table 1).

Most authors are unanimous that with respect to micropropagation - the number of newly formed buds, nodes and shoots, MS full strength medium resulted in the best response compared to half and quarter strength medium (Dahab *et al*., 2005; Hidayah *et al*., 2012). However ½ MS medium was more effective for seedling development of *Thymus satureioides* Coss (Aicha *et al.*, 2013) and for shoot regeneration of *Mentha spicata* compared to full strength MS medium (Fadel *et al*., 2010).

B. Effect of medium salt strength on shoot growth

In Table 1 are presented the results on the effect of different MS medium salt strength on fresh weight (FW) and dry weight (DW) of proliferated shoots. The shoots growth was significantly reduced with decreased of medium salt strength after four weeks of culture. When the shoots were cultured on full strength MS medium, the highest FW (1.66 g) and DW (0.25 g) were measured. The lowest FW (1.04 g) and DW (0.12 g) were recorded, when the shoots grow on ¼ MS medium. The results showed that the higher concentrations of macro salts and vitamins (full MS medium, follow by ½ MS medium) were more effective for the shoot growth and biomass accumulation than the lower level of macroelements used. The effect of the concentration of mineral elements in the medium on plant growth is closely related to the uptake of mineral elements from the medium (Shohael *et al*., 2013). The synergistic effect of auxin and cytokinin used in the combination led to a considerable increase in the mean shoot number, shoot fresh and dry weights per explant (Table 1). In the present study, FW and DW were found to be the best on the full MS medium. This medium was determined to be optimal for maximum biomass production.

C. Effect of medium salt strength on in vitro *rooting efficiency*

The decrease of macro- and micro salts by half in the nutrient medium usually improves rhizogenesis of some medicinal plant species (Baskaran and Jayabalan, 2005; Trejgell *et al.*, 2009). Less studied are media contained third and quarter contents of MS nutrients. The strength of the culture medium had a significant effect on root formation. In the current study, MS salts and vitamins levels with auxin IBA (0.5 mg L^{-1}) was found to affect in vitro rooting of *A. montana* (Table 2, Fig. 1b). The highest number of roots per plant (13.55) was achieved on quarter strength MSR4 medium supplemented with 0.5 mg L^{-1} IBA, as the roots had also the highest mean length (2.81 cm) compared to other examined media. The plants grown on this medium were with the lowest mean height (1.18 cm). No significant difference was observed in values of studied parameters (number of roots per plants and length of roots) among half (MSR2) and third (MSR3) strength nutrient media. However, they were with well-developed overground part (1.82 - 2.02 cm height) allowing them to more easily adapt to the ex vitro conditions.

Table 2. Effect of MS medium salt strength on the in vitro rooting of *A. montana* after four weeks of culture.

Nutrient media with 0.5 mg L^{-1} IBA	Height of plant, cm	Number of roots per plant	Length of root, cm
Full strength (MSR1)	2.55 ± 0.14^{c}	6.2 ± 0.49^{a}	0.48 ± 0.07^{a}
½ (MSR2)	2.02 ± 0.11^{b}	10.6 ± 1.02^{b}	1.36 ± 0.17^{b}
1/3 (MSR3)	1.82 ± 0.12^{b}	11.0 ± 0.82^{b}	1.75 ± 0.18^{c}
¼ (MSR4)	1.18 ± 0.10^{a}	13.55 ± 1.06^{c}	2.81 ± 0.22^{d}
LSD	0.22	1.65	0.31

The data are presented as means of 20 plants per treatment ± standard error. Different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

Full strength rooting MSR1 medium was less effective for root induction of *A. montana.* The most of the researches reported that ½ MS medium promotes rhizogenesis of in vitro shoots in the greatest degree compared to full and quarter strength nutrient medium (Dahab *et al.*, 2005; Fadel *et al*., 2010; Aicha *et al*., 2013). Induction of adventitious roots of *A. montana* micropropagated plants were inducted successfully on ½ MS medium containing IBA (Le, 2000), while Weremczuk-Jeżyna and Wysokińska (2000) achieved shoot rooting in full MS medium without the addition of the auxin. The root number and roots length of *S. rebaudiana* Bertoni plants regenerated from callus culture was significantly influenced by strength of MS medium and concentrations of IBA. Maximum rooting of regenerated plants were observed on ¼ strength MS medium supplemented with 0.1 mg L^{-1} IBA (Patel and Shah, 2009). In the rooting stage, *Mentha spicata* shoots cultured on ½ MS produced a large number of roots than when grown on full MS medium (Fadel *et al*., 2010). In the present study, among the different MS salts concentrations tried, ½ strength MS medium with 0.5 mg L^{-1} IBA was found suitable in terms of mean plant height, root number and root length for the subsequent ex vitro acclimatization. The IBA seems to be the best auxin in *A. montana* to the initiation of the root induction.

The shoots rooted on ½ MS medium were transferred to mixure (peat: perlite: coconut fiber, 2:1:1 v/v/v) for ex vitro acclimatization. This mix had beneficial effect on survival and growth of the plants, as described earlier (Petrova *et al.*, 2011). In vitro propagation is often restricted by high percentage of plant loss, when transferred from in vitro to ex vitro conditions (Pospóšilová *et al.*, 1999). Available in well-developed root system of micropropagated plants and controlled reduction of humidity provided rapid ex vitro acclimatization after two months with 90% survival. Furthermore, the plants continue to grow and form new leaves, which is a sign of successful ex vitro adaptation (Fig. 1c). All plants were successfully transferred to greenhouse conditions with 60-65 % survival.

D. Effect of medium strength on the total phenolic content and antioxidant activities of shoot, derived from micropropagation and rooting stages

Polyphenol synthesis and accumulation in plants is generally stimulated in response of abiotic or biotic strsess (Cheynier *et al*., 2013), such as excess or deficiency of nutrients. In the present study, total phenolic content was higher in shoots derived from rooting stage compared to these from micropropagation stage. The highest average phenolic content was recorded in shoots grown on ¼ MS rooting medium (112.52 mg GAE/g extract) (Fig. 2).

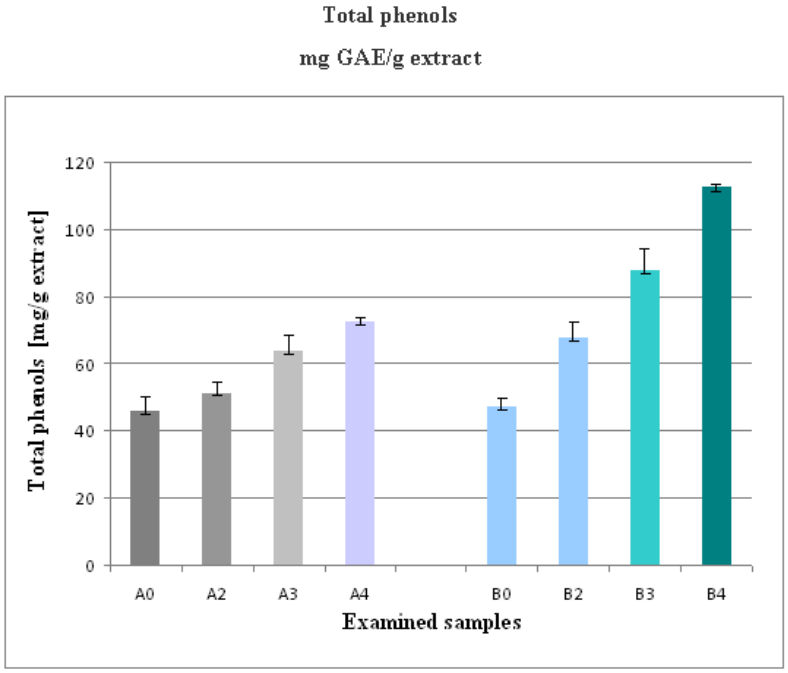

Fig. 2. Total phenolic content of *A. montana* depending on the strength of the MS nutrient medium. Shoots derived from micropropagation stage: A0 - full strength medium (MSP1); A2 - ½ (MSP2); A3 - 1/3 (MSP3); A4 - ¼ (MSP4); Shoots derived from in vitro rooting stage: B0 - full strength media (MSR1); B2 - ½ (MSR2); B3 - 1/3 (MSR3); B4 - ¼ (MSR4).

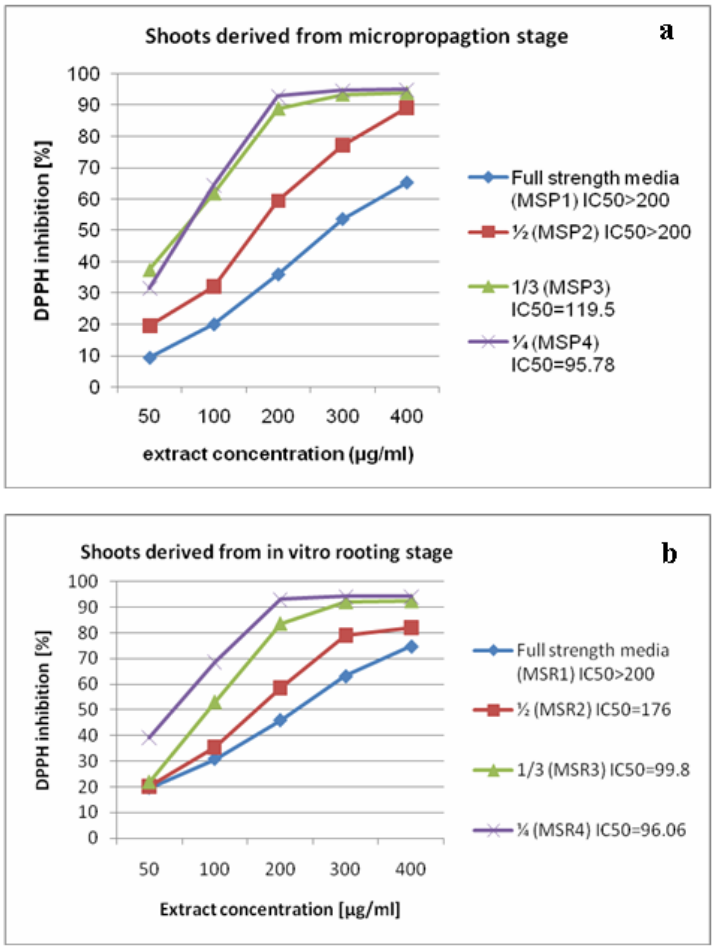

Fig. 3. (a). Free radical scavenging activity of studied samples. Shoots of *A. montana* derived from micropropagation stage (b). Shoots derived from in vitro rooting stage.

There is an inverse relationship between the strength of the medium and the total phenolic content. The plants grown on ¼ MS strength medium were with the highest phenolic content, while those grown in full strength medium had the lowest phenolic content, whether derived from micropropagation or from in vitro rooting stage. Similar observations were made by Fadel *et al.* (2010) and by Alturki *et al.* (2013) in the analysis of the effect of different strength of medium on organogenesis, phenolic accumulation and antioxidant activity of spearmint (*Mentha spicata*) and Date Plam (*Phoenix dactylifera* cultivars).mAccording them full strength media predominantly promote primary metabolism and cellular growth, but sometimes hampered the morphological and biochemical tissue differentiation. MS salt strengths had no significant effect on the contents of phenols in shoots of *Ruscus hypoglossum* L., but significantly influenced the content of chlorophyll A and carotenoids (Dahab *et al.*, 2005), which had the highest values when ½ and ¼ strength MS media were used. Methanol extracts of studied samples were examined for their antiradical properties using a DPPH assay and expressed as IC50 value – extract concentration providing 50% inhibition of DPPH solution (Fig. 3). The highest antiradical properties were determined for the shoots cultured on media contained quarter (1/4) strength MS micro-, macro- nutrients and vitamins as IC50 values were respectively - 95.78 µg/ml for micropropagated shoots (Fig. 3a) and 96.06 µg/ml for in vitro rooted shoots (Fig. 3b). The plants grown on 1/3 MS nutrient medium showed also high antioxidant activity - IC50 = 119.5 and 99.80 µg/ml. The extracts of shoots cultured on full strength media for micropropagation or rooting had low activity and their IC50 values were above 200 µg/ml. Higher activity of the extracts of the samples corresponds to a high content of phenols in them. The positive correlation between the total

phenolic content and DPPH radical-scavenging activity has been reported by many authors (Farasat *et al*., 2014; Pal *et al*., 2015). Decreased salt concentration resulted in osmotic stress and induces the production of antioxidant metabolites.

CONCLUSSION

The results showed that MS strength media played a significant role of micropropagation frequency, rooting efficiency, phenols accumulation and antioxidant activity of *A. montana*. The full MS medium supplemented with 1 mg L^{-1} BAP and 0.1 mg L^{-1} IAA significantly enhanced the shoot multiplication and biomass accumulation compared to the other medium strengths. The root induction was affected by MS medium salt strength. The propagated plants were successfully hardened and transferred to greenhouse conditions. Lower MS medium strength was found better for total phenolics and flavonoid accumulation. The shoots, derived from in vitro rooting stage, grown on ¼ strength of MS medium had higher total phenolic content compared to these from micropropagation stage. The current study show that the concentration of MS salts and vitamins can be optimized to enhance antioxidant metabolites of *A. montana*. The data contribute for further more extensive studies of the biologically active compounds of in vitro cultures of this valuable species depending on the nutritive factors.

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