MICROPROPAGATION AND *EX SITU* CONSERVATION OF *ACHILLEA THRACICA* VELEN.

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ABSTRACT

The genus *Achillea* (*Asteraceae*) comprises more than 130 perennial species most of which grow wild in Northeastern Europe and Northwestern Asia. In this study, *Achillea thracica* Velen. (*in vivo* plants) was collected at its natural habitat in Thracian lowland, Manole village, Plovdiv, Bulgaria, and propagated successfully *in vitro* on standard cultivation MS medium enriched in Gamburg vitamins (MS_{B5} medium). The plants were successfully *ex vitro* adapted and transferred to the experimental field in Lozen mountain. *A. thracica* is a Bulgarian endemic species containing a variety of biologically active compounds: monoterpenes, sesquiterpenes and flavonoids. The aim of this study was to investigate the content of total phenols and flavonoids and the total antioxidant activity in *in vivo, in vitro* and *ex vitro* adapted plants. Significant total phenolic content was observed in *in vivo* and *ex vitro* grown plants (48.02±4.06 and 40.02±0.49 mg gallic acid (GA).g^{-1} dry weight (DW), respectively). Interestingly, a higher content of flavonoids was observed in *in vitro* and *ex vitro* plants (10.62±0.28 and 12.49±0.46 mg quercetin (Q).g^{-1} DW, respectively). On the other hand, significant increase in the total antioxidant activity was observed in *in vitro,* followed by *in vivo* plants (83.39±0.54 and 73.18±2.3 mM tocopherol.g^{-1} DW, respectively). The present work offers an effective alternative method for propagation and preservation of the important medicinal plant *A. thracica*, and in parallel the modulation of its biological activity was followed during the different cultivation conditions.

Keywords: *in vitro propagation, ex situ conservation, antioxidant activity, medicinal plant*

Introduction

The genus *Achillea* (family *Asteraceae*) are perennial herbs, most of which grow wild in Northeastern Europe and Northwestern Asia and comprise more than 130 species. *Achillea* sp. are medicinal plants that possess anti-inflammatory, antioxidant and antimicrobial properties (Cavalcanti et al. 2006, Stojanovic et al. 2005). *Achillea thracica* Velen. is an endangered Bulgarian endemic plant (Stanev et al. 2011) under the Bulgarian Biodiversity Act and the Bern Convention protection and it is included in the European Red List of Vascular Plants (Bilz et al. 2011). The leaves of *A. thracica* contain variety of biologically active compounds: monoterpens, sesquiterpens, phenolic acids and flavonoids (Saukel et al. 2003).

The plant micropropagation is an approach that ensures a rapid multiplication and preservation of the genetic potential of the initial plants. In this respect, *A. thracica* as being an endangered plant species is a suitable subject for *in vitro* cultivation, *ex vitro* adaptation, and *ex situ* conservation. The aim of this study was to investigate in particular the possible changes in the total phenolic content and antioxidant activity of *A. thracica* plants during their *in vitro* propagation and *ex vitro* adaptation compared to the *in vivo* plants.

Material and methods

Plant material

Fully grown matured plants *Achillea thracica* Velen. (*in vivo*) were collected at its natural habitat in Thracian lowland, Manole village, near Plovdiv, Bulgaria and propagated successfully *in vitro* on MS medium (Murashige and Skoog, 1962) with Gamburg vitamins (MS_{B5} medium). The plants were

successfully *ex vitro* adapted and transferred to the experimental field in Lozen mountain. The plant material was collected and was dried and ground in a grinder.

Determination of total phenolic content

Total phenolic content in the crude extracts of *E. officinalis* L. were determined according to Singleton et al. (1999) with modifications. Aliquots of 0.1 ml of chloroform and methanol extracts with concentration 10 mg.ml^{-1}, were mixed with 1.5 ml Folin–Ciocalteu reagent (1:10 diluted in dH2O) and 1.4 ml 7.5 % sodium carbonate. The samples were incubated at room temperature for 30 min and after the absorbance was read at 765 nm by using Shimadzu UV 1800 spectrophotometer. The total phenolic content was expressed as mg Gallic acid (GA).g-1 dry weight (DW).

Determination of total flavonoid content

Total flavonoid content was measured according to Chang et al. (2002) with slight modifications. Aliquots of 0.5 ml of extract with concentration 10 mg.ml^{-1}, were mixed with 1.4 ml methanol, 0.1 ml 10 % aluminum chloride, 0.1 ml 1 M potassium chloride, and 2.8 ml distilled water. The mixture was incubated at room temperature for 30 min and after the absorbance was measured with a Shimadzu UV 1800 spectrophotometer at 415 nm. Total flavonoid content was expressed as mg Quercetin (Q).g-1 DW.

Determination of total antioxidant activity

The total antioxidant capacity of the extracts was evaluated by the method of Prieto et al. (1999) with slight modifications. The antioxidant capacity of the extracts was measured spectrophotometrically using a phosphomolybdenum method, based on the reduction of Mo(VI) to Mo(V) by the sample analyzed, and the subsequent formation of green phosphate/Mo(V) compounds with a maximum absorption at 695 nm. A 0.25 ml aliquot of sample solution (10 mg.ml^{-1}) was mixed with 2.5 ml of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each sample was measured at 695 nm, against a blank solution by using a Shimadzu UV 1800 spectrophotometer. A blank solution contained 2.5 ml of reagent solution and the appropriate volume of methanol used for the dissolution of the samples and it was incubated under the same conditions as the other samples. The total antioxidant capacity was expressed as equivalents of mM α-tocopherol.g-1 DW.

Statistical analysis

Presented data for all experiments are average values from at least four independent experiments and are compared by standard error of the means (S.E.M.). The statistical significance between the growth variants (*in vivo*, *in vitro*, *ex vitro*) was assessed by using *t*-test at $P \leq 0.01$.

Results and discussion

The highest total phenolic content was observed in *in vivo* and *ex vitro* grown plants (48.02±4.06 and 40.02±0.49 mg GA.g^{-1} DW, respectively) compared to *in vitro* plants (19.50±1.23 mg GA.g^{-1} DW) (Fig. 1). It is possible that the *in vitro* growth affected the synthesis of phenolic compounds because the plants developed in highly controlled environment. A significant, nearly twice, increase in the content of total flavonoids was observed in *in vitro* and *ex vitro* plants (10.62±0.28 and 12.49±0.46 mg Q.g^{-1} DW, respectively), compared to *in vivo* plants (6.84±0.58 mg Q.g^{-1} DW) (Fig. 2). The reason for this interesting observation could be the effect of growth conditions that causes the accumulation of protective compounds for better adaptation. The highest antioxidant potential was established in *in vitro*, followed by *in vivo* grown *A. thracica* (83.39±0.54 and 73.18±2.3 mM tocopherol.g^{-1} DW, respectively), whereas the antioxidant activity of the *ex vitro* grown plants was reduced to 51.12±2.93 mM tocopherol.g^{-1} DW. The results suggest that the *in vitro* cultivation probably caused stress to the *A. thracica* plants (Pereira et al. 2013), while during the following *ex vitro* growth the plants became with lower antioxidant activity that

could be due to improved environmental acclimation. Whether there is a link between the flavonoid content and the antioxidant activity should be further elucidated.

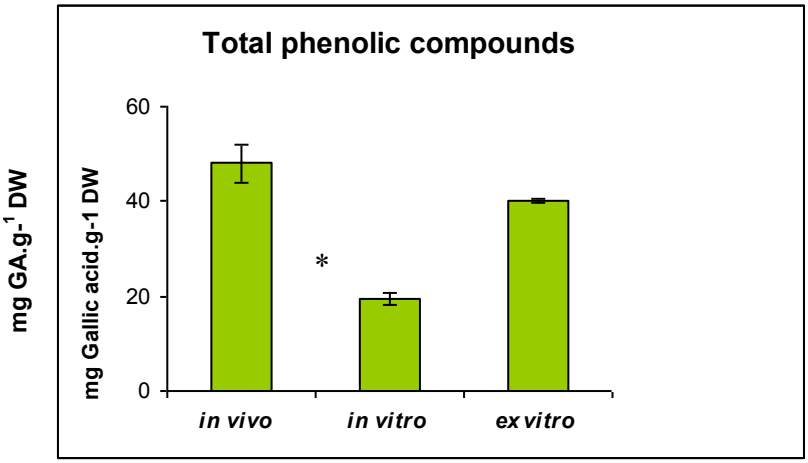

Fig. 1. Concentration of total phenolic compound in *in vivo, in vitro* and *ex vitro A. thracica* plants. Significant changes compared to *in vivo* control are indicated with asterisk - *, $P<0.01$.

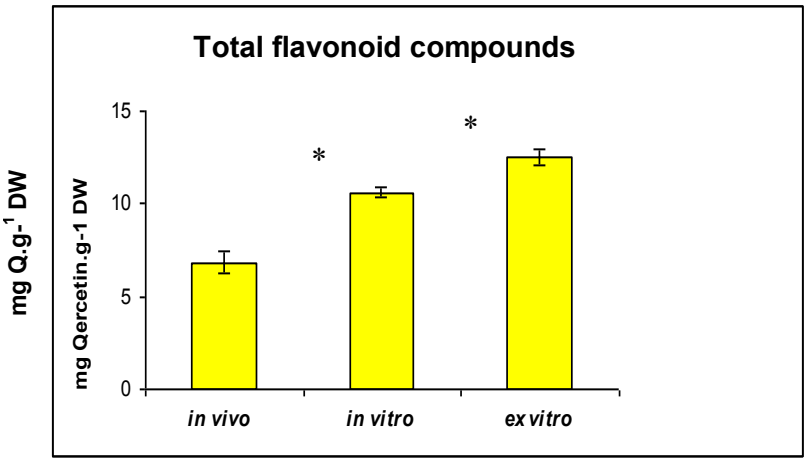

Fig. 2. Concentration of total flavonoid compound in *in vivo, in vitro* and *ex vitro A. thracica* plants. Significant changes compared to *in vivo* control are indicated with asterisk - *, $P<0.01$.

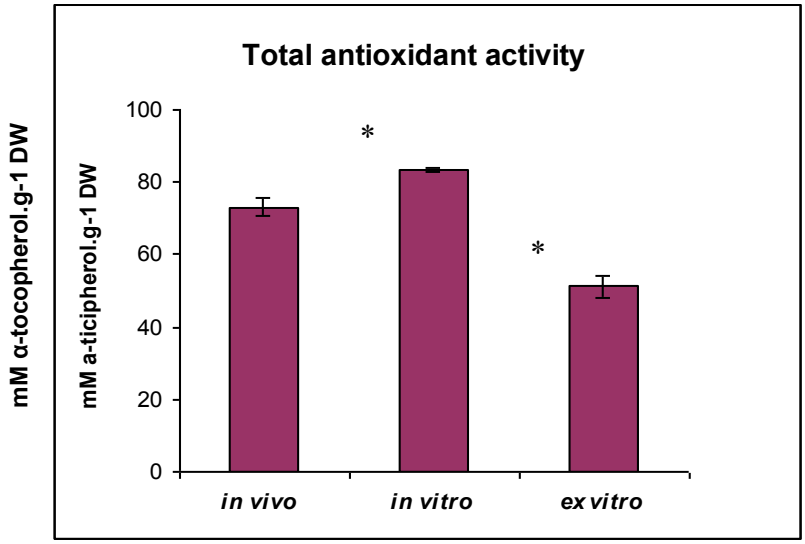

Fig. 3. Concentration of total antioxidant activity in *in vivo, in vitro* and *ex vitro A. thracica* plants. Significant changes compared to *in vivo* control are indicated with asterisk - *, $P<0.01$.

Conclusion

In this work we offer an effective alternative method for propagation and preservation of the important medicinal plant *A. thracica*. Further experiments with extracts are needed to determine the optimal concentration of secondary metabolites of *A. thracica* for different purposes as accumulation of secondary metabolites with high antioxidant capacity. These experiments could be essential for further pharmacological, physiological and biochemical studies of secondary metabolites.

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