Bulgarian Journal of Agricultural Science, 19 (2) 2013, 57–60
Agricultural Academy

Effect Of 6-Benzylaminopurine On Micropropagation Of *Artemisia Chamaemelifolia* Vill. (*Asteraceae*)

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Abstract

HRISTOVA, L., E. DAMYANOVA, Z. DOICHINOVA and V. KAPCHINA-TOTEVA, 2013. Effect of 6-benzylaminopurine on micropropagation of *Artemisia chamaemelifolia* Vill. (*Asteraceae*). *Bulg. J. Agri. Sci.,* Supplement 2, 19: 57–60

The influence of different concentrations (0.1–1.0 mg.L^{-1}) of the cytokinin 6-benzylaminopurine (BA) on the *in vitro* propagation of the endangered aromatic plant *Artemisia chamaemelifolia* Vill. (*Asteraceae*) was examined. The increase of concentration stimulated the formation of shoots but shortened their length. The middle concentrations of 0.5, 0.6 and 0.7 mg.L^{-1} BA gave the highest number of shoots but they also appeared to promote negative effects like vitrification and necrosis more than the other variants. Although there was no clear trend, all variants had higher fresh weight and formed much more primary callus than the control explants grown on Murashige & Skoog medium with Gambourg vitamins. Over 50 % of the explants gave rise to more than 10 new shoots per explant at concentrations of 0.2, 0.3 and 0.9 mg.L^{-1} BA. All concentrations of BA suppressed root formation and manifested different extent of vitrification. This is the first report on the influence of BA on the micropropagation and growth of *Artemisia chamaemelifolia* Vill. The results obtained are standpoint for further biotechnological application for biomass and valuable metabolite production.

Key words: *in vitro* propagation, endangered species, cytokinin
Abbreviations: *FW* – fresh weight; DW – dry weight; BA – 6-benzylaminopurine or benzyl adenine

Introduction

Artemisia chamaemelifolia Vill. (*Asteraceae*) is an aromatic perennial, critically endangered species from the Red Data Book of Bulgaria. The single, small and low-reproductive population of this glacial relict in Ponor Mountain (West Bulgaria) is threatened by aridization and tourism (EuroMed Plantbase; Gussev, 2011). The status of the species and the lack of data about its active substances make it worth exploring. There is a Bulgarian report on silphiperfolane sesquiterpenes (Trendafilova-Savkova et al., 2003), a publication about the essential oils of plants from Iranian populations (Morteza-Semnani et al., 2008) and a research on the antiviral activity of the species (Khajeh Karamoddini et al., 2011), all based on *in vivo* gathered material. We recently reported of the initiation of *in vitro* cultures from ripe seeds of *A. chamaemelifolia* (Hristova et al., 2012) but still now, there are no data for the micropropagation of this plant as well as on the effect of the growth regulator BA.

In vitro cultures could promote research of the metabolites and potential medicinal properties of this species because field-grown plants can be affected by seasonal and somatic variations, bacterial and fungal infestation, as well as environmental pollution that might change the medicinal value of the plants (Geng et al., 2001). Harvesting medicinal plants on a large scale from their natural habitats causes a depletion of plant resources. That is why the germplasm conservation of these valuable genotypes is imperative and shoot culture propagation, utilized to maintain clonal fidelity would be of high value (Sen and Sharma, 1991; Murch et al., 2000). On the other hand, the metabolite content can be modified to obtain highly productive plants by varying the *in vitro* conditions and ingredients of the culture medium (Kirakosyan et al., 2004; Arencibia et al., 2008). Even though there are several reports on other species of the genus *Artemisia* (Mackay and Kitto, 1988; Mathe and Laszloffy, 1991; Nin et al., 1996; Sharief et al., 1997; Mozetti and De-Donato, 1998; Saxena, 2001; Liu et al., 2003), no reports on the systematic

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cultivation and tissue culturing of *A. chamaemelifolia* are available at present.

In this context, the aim of the present study was to investigate the influence of a range of concentrations (0.1–1.0 mg.L^{-1}) of the cytokinin 6-benzylaminopurine (BA) on the *in vitro* propagation of the endangered and potentially medicinal aromatic plant *A. chamaemelifolia.*

Materials and Methods

As an initial material were used ripe dry seeds of *Artemisia chamaemelifolia* Vill., gathered from the locality of Breze village in Ponor mountain, altitude ≈ 1560 m.

The seeds were successively exposed to cold stratification (4°C, in dark, for 4, 8 and 16 months) and sterilization (presterilization in 70% ethanol, 1–2 min; sterilization in 100% Domestos® with 4–5% NaOCl (4.8 g/100 ml), 20 min; triple soaking and rinsing with sterilized distilled water). They were germinated in watered agar, 8 g/l agar, pH = 7.75 – 7.8, in test tubes, in dark, at room temperature (18–21°C).

The obtained seedlings were transferred on a plant regeneration medium after onset of photosynthesis. We used plain MS formulation (Murashige and Skoog, 1962), supplied with Gambourg (B5) vitamins, 30 g/l sucrose, 8 g/l agar, pH = 7.75–7.8, at 16/8 h light period and 23±1°C. The subculture on fresh medium was carried every 30 days (1-month passage) until we obtained regenerated plantlets with roots and more than two nodes per plantlet. The cultivation period was two months.

In vitro shoot cultures were induced from mono-nodal stem segments excised from the regenerated plantlets. Explants from regenerated plants (shoots with two axillary buds) were propagated on MS medium supplemented with 0.1–1.0 mg.L^{-1} 6-benzyladenine BA. The study was carried out in three independent experiments with 5 replicates per variant. Each replicate contains 8 explants (in total 40 explants per variant).

The presented data for all experiments are average values from the three independent experiments and are compared by standard error of the means (S.E.M.).

Results and Discussion

Aseptically growing shoots were induced from mono-nodal stem segments on MS medium supplemented with different concentration of BA (0.1–1.0 mg.L^{-1}) to evaluate the effect of this cytokinin on *A. chamaemelifolia* shoot multiplication. Shoots were developed directly from all the non-necrotic explants within 4 weeks, on all media variants. The study clearly showed an increase of the number of shoots and decrease of their length (Figure 1A, B) with the addition and increasing of BA concentration in the culture medium. The observed decrease in length of shoots at high concentrations of BA (0.4–1.0 mg.L^{-1}) might be due to the inhibition of organogenesis and induction of callusogenesis (Dimitrova et al., in press). Three medium variants promoted strong multiple-shoot development (more than 10 small shoots per explant were visualized but they were not taken into account when presenting the average number of shoots) and more than 50% of the explants at 0.3, 0.4 and 0.9 mg.L^{-1} BA formed multiple shoots along with the main shoots (Figure 2B, Table 1). The effect of BA on stimulating shoot formation was reported for other species from the genus *Artemisia* (Holobiuc and Blindu, 2007; Zia et al., 2007).

There was a trend of increase of the fresh weight of the *in vitro* propagated explants (FW) along with the increase of the BA concentration (Table 1). However, this might be due to hyperhydracity caused by vitrification, especially at 0.4 mg.L^{-1} BA where the weight of the explants reduced drastically up to 11

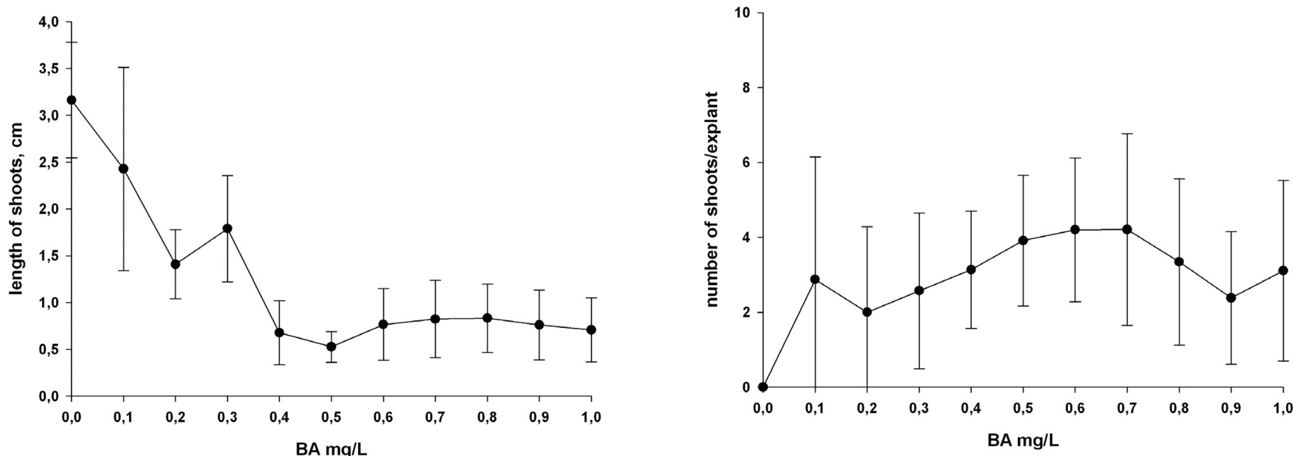

Fig. 1. Influence of different concentrations of BA (0.1–1.0 mg.L^{-1}) on the length (cm) [A] and number of shoots per explant [B]. Error bars represent standard error of the means (average deviation)

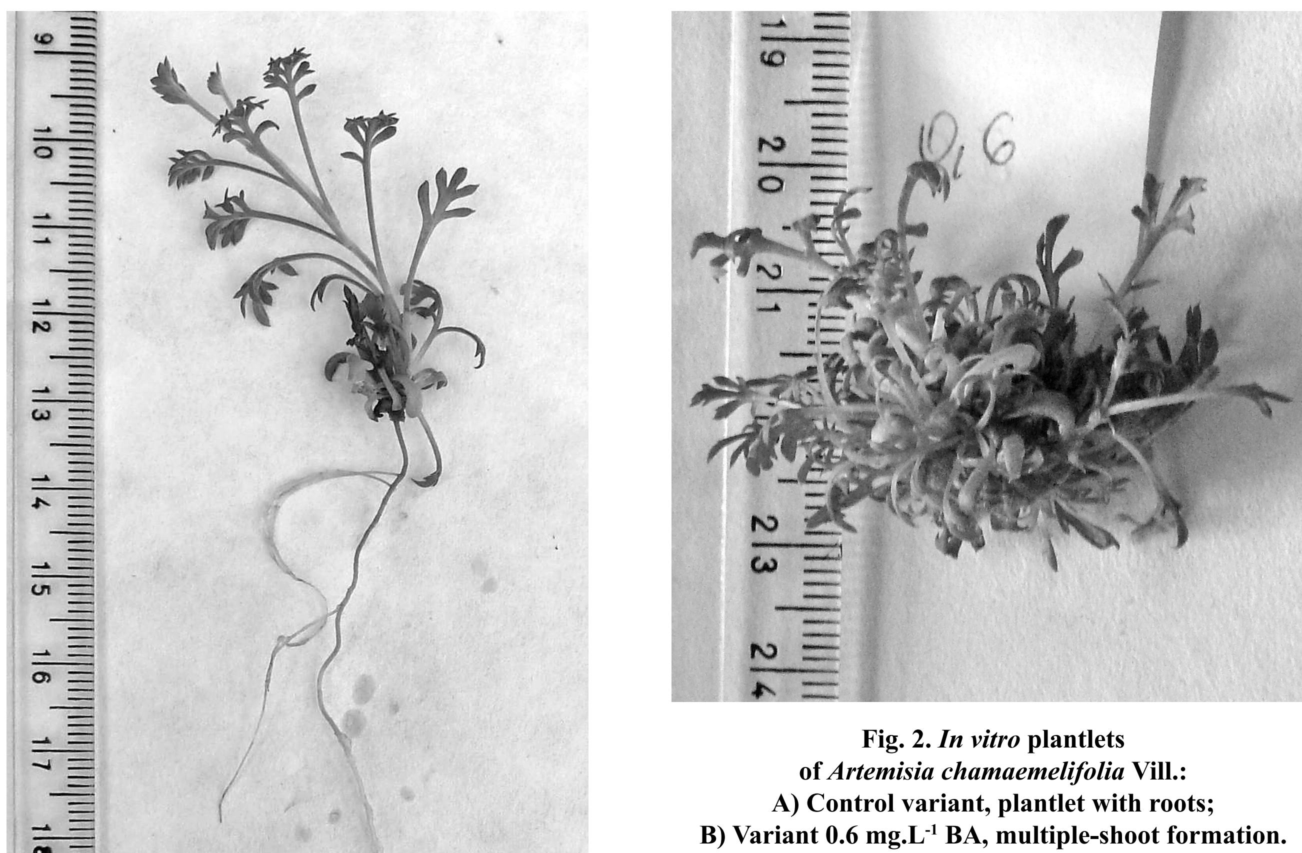

Fig. 2. *In vitro* plantlets of *Artemisia chamaemelifolia* Vill.: A) Control variant, plantlet with roots; B) Variant 0.6 mg.L^{-1} BA, multiple-shoot formation.

Table 1

Influence of different concentrations of BA (0.1–1.0 mg.L^{-1}) on FW, length and number of shoots, root formation, callusogenesis, vitrificated explants per variant and number of necrotised and undeveloped explants of *in vitro* propagated *Artemisia chamaemelifolia* Vill.; legend: + weak callus formation, ++ good callus formation, +++ very strong callus formation, +* root formation, - no roots

Variants	Average FW/ explant [mg]	Multiple-shoot formation %	Degree of primary callus formation	Root formation	FW per variant [mg]	DW per variant [mg]	Vitrification %	Number of necrotised and undeveloped explants
control	0.065 ± 0.013	0	+	+*	0.331	0.050	13	0
BA 0.1 mg.L^{-1}	0.137 ± 0.054	8	+++	–	1.636	0.200	8	14
BA 0.2 mg.L^{-1}	0.138 ± 0.064	21	+++	–	0.902	0.118	7	13
BA 0.3 mg.L^{-1}	0.209 ± 0.099	50	+++	–	1.313	0.139	36	15
BA 0.4 mg.L^{-1}	0.203 ± 0.121	53	++	–	4.173	0.390	33	13
BA 0.5 mg.L^{-1}	0.151 ± 0.070	26	++	–	2.031	0.272	13	19
BA 0.6 mg.L^{-1}	0.234 ± 0.179	29	++	–	1.628	0.204	48	20
BA 0.7 mg.L^{-1}	0.190 ± 0.118	26	+++	–	2.261	0.258	53	21
BA 0.8 mg.L^{-1}	0.168 ± 0.098	38	+++	–	3.24	0.378	38	14
BA 0.9 mg.L^{-1}	0.301 ± 0.228	52	++	–	2.934	0.354	43	14
BA 1 mg.L^{-1}	0.201 ± 0.098	44	+++	–	3.126	0.330	48	13

times folds after drying (Table 1). The vitrification negatively affects the leaves causing abnormal morphology and physiology (Ziv, 1991) and this leads to ineffective micropropagation in long-term perspective. Another negative aspect was the risk of necrosis and retained development of the explants. Variants with 0.5, 0.6 and 0.7 mg.L^{-1} BA showed such an influence over half of the set explants (Table 1).

All the variants and the control formed primary callus at the

base of the shoots. Callus can be induced either by injury or by the addition of growth regulators – usually a proper ratio of auxin and cytokinin (Sugimoto et al., 2011). In our case, even the lowest concentration of BA applied alone caused callogenesis (Table 1). Therefore, we suggested it is probably an inherited ability of the species to form callus and a genotype dependent feature. The callus is the starting material for cell suspension cultures, which are widely used for bioreactor cultivation of plants (Paniego and Giulietti, 1994), and this is a premise for expanding our study into application of lab and large scale bioreactor systems.

The used BA concentrations have suppressed root formation and could not be applied for whole-plant regeneration (Table 1).

Conclusions

Artemisia chamaemelifolia Vill. can be successfully propagated *in vitro* from stem explants by the addition of low concentration of BA (0.1–0.3 mg.L^{-1}) to the solid MS culture medium. The *in vitro* cultivation of the species can provide rapid propagation of plant biomass with preservation of it biosynthetic activity level. The callusogenesis observed in all variants including also the control is a prerequisite for induction of cell cultures of *A. chamaemelifolia* suitable for biomass and biologically active compounds production in bioreactor. This study is an initial survey that needs to expand towards a rapid and continuous year-round supply of large number of disease-free plants for different purposes (*ex situ* collection, biomass), as well as for proper chemical profiling of the metabolites of this species.

Acknowledgements

The research work is supported by project # 22/2012 National Science Fund – Sofia University "St. Climent Ohridski". We are grateful to Dr. Chavdar Gussev from IBER – Bulgarian Academy of Sciences for the gathered seeds of *A. chamaemelifolia*.

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