

## STUDIES ON THE *IN VITRO* MICROPROPAGATION ABILITY OF *ARONIA MELANOCARPA* (MICHX.) ELLIOT

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### Abstract

Since the expression of a high ability for *in vitro* regeneration and proliferation is a very important condition for any biotechnological approach for clonal propagation, the influence of genotype and culture medium composition on the number of shoots regenerated and their length in successive subcultures was investigated in *Aronia melanocarpa* (Michx.) Elliot cultivars 'Melrom' and 'Nero'. Chokeberry cultivar 'Nero' showed a significantly higher ability of regeneration compared to the cultivar 'Melrom', the greatest number of shoots being obtained with the basal medium containing MS macroelements, LF microelements and LF vitamins, supplemented with 4,5 mg×dm<sup>-3</sup> BA and 0,6 mg×dm<sup>-3</sup> IBA.

Keywords: *Aronia melanocarpa*, axillary shoots, culture medium, genotype.

### 1. INTRODUCTION

From its spread in Europe, in the middle of the last century, *Aronia melanocarpa* (Michx.) Elliot was remarked, especially, by high content of phenolic compounds and carotenoids in its fruits, but also by their richness in dietary fibers, proteins, minerals, vitamins, and organic acids (Lehmann, 1990; Mayer-Miebach et al., 2008; Oszmianski and Wojdylo, 2005; Tanaka and Tanaka, 2001; Wawer et al., 2006), which are assigned a variety of protective and curative properties. Berries, juice or extracts of *A. melanocarpa* (Michx.) Elliot were the subject of numerous studies aimed at highlighting their potential health benefits. Thus, from the researches on the antioxidant (Wu et al., 2004; Oszmianski and Wojdylo, 2005; Seeram et al., 2008) hepatoprotective (Kowalczyk et al., 2003; Valcheva-Kuzmanova et al., 2004), cardioprotective (Bell and Burt, 2007) and hypoglycaemic effects (Simeonov et al., 2002), to those on antimutagenic (Duthie et al., 2007) and antitumoral effects (Bermudez-Soto et al., 2007), or those on protective action against degenerative diseases, the scientific literature is rich in information outlining their prophylactic and therapeutic properties, without suggesting any unwanted or side effect of their use (Kokotkiewicz et al., 2010). *In vitro* micropropagation represents most often a quick, economical and safe technique for multiplication of valuable varieties (cultivars) of trees and shrubs. Since 1990s, various cultivars of *A. melanocarpa* (Michx.) Elliot, popular for their horticultural characteristics, were tested for their *in vitro* micropropagation ability (Brand and Cullina, 1992; Litwińczuk, 2002; Litwińczuk, 2013; Kwak et al., 2015). Among the most popular varieties of *A. melanocarpa* (Michx.) Elliot are

'Nero', 'Rubina', 'Viking', 'Kurkumäcki', 'Hugin', 'Fertödi' and 'Aron' (Strigl et al., 1995, McKay, 2004, cited by Kulling and Rawel, 2008).

The main objective of our studies was to determine the *in vitro* regeneration and proliferation ability of the Romanian chokeberry cultivar 'Melrom' (released in 2016), compared to cultivar 'Nero'. In order to optimize the *in vitro* micropropagation process was analyzed the influence of the culture medium composition on the number of shoots regenerated and their lengths in successive subcultures.

## 2. MATERIAL AND METHODS

The explants from 'Nero' and 'Melrom' cultivars of *A. melanocarpa* (Michx.) Elliot tested for their ability of regeneration and proliferation *in vitro* were obtained from field-grown plants in the small fruits collection of the Research Institute for Fruit Growing, Pitești-Mărăcineni.

The cultivar 'Nero', very popular, produce berries which remain on the plant until the late autumn, with a long storage life. 'Melrom' is a Romanian black chokeberry cultivar created at the Research Institute for Fruit Growing Pitești-Mărăcineni, and released in 2016. This cultivar has morphological characteristics similar to that of 'Nero' cultivar, but distinguishes by its larger fruits.

***In vitro culture initiation.*** In april 2016, branches of *A. melanocarpa* (Michx.) Elliot, cultivars 'Nero' and 'Melrom', were harvested from the field-grown plants and pre-sterilized by washing in tap water to which 2-3 drops of Domestos were added. Subsequently, lateral buds were disinfected successively with 96% ethanol for 5 minute and with 6% calciumhypochlorite for 10 minutes. After sterilization, the biological material was rinsed in three baths of distilled water.

Shoot apices with 2-3 leaf primordia and sizes of 0,1 - 0,3 mm were excised from the sterilized foliar buds and distributed individually into Pyrex tubes of 20-25 cm<sup>3</sup>, containing 10 ml of culture medium sterilized by autoclaving. For this stage, the culture medium has been solidified as an inclined plane.

The culture medium containing Lee-Fossard macroelements, microelements and vitamins, 20 g×dm<sup>-3</sup> sucrose, 0,1mg ×dm<sup>-3</sup> giberellic acid (GA<sub>3</sub>) and 1mg ×dm<sup>-3</sup> benzyladenine (BA) was solidified with 7g/l agar. The pH of the culture medium was adjusted to 5,7 with 0,1N KOH before autoclaving for 20 minutes at 121 °C.

***Micropropagation by axillary shoot proliferation.*** Microshoots obtained after the initiation of *in vitro* culture were divided and transferred into Ehrlenmayer flasks of 100cm<sup>3</sup> capacity, containing 30 ml of culture medium composed of Murashige-Skoog (MS) macroelements, Lee-Fossard (LF) microelements and Linsmaier-Skoog (LS) vitamins (Table 1), 40g×dm<sup>-3</sup> dextrose and solidified with 8g×dm<sup>-3</sup> agar. For each of the two cultivars investigated for their micropropagation ability, were tested culture media optimized by supplementation with 0,3 mg×dm<sup>-3</sup> GA<sub>3</sub>, BA in concentration of either 3 mg×dm<sup>-3</sup> or 4,5 mg×dm<sup>-3</sup>, and IBA in concentration of either 0,3 mg×dm<sup>-3</sup> or 0,6 mg×dm<sup>-3</sup>. The experimental treatments with various combinations and concentrations of growth regulators are shown in Table 1. The culture flasks were sealed with plugs of cotton wrapped in aluminium foil and then autoclaved. The same treatments were used in three successive subcultures of 'Nero' and 'Melrom' shoots.

Shoot apices culture and microshoots cultures, respectively, were incubated in a growth chamber at 22-24°C, under a photoperiod of 16 hours light /8 hours darkness, and a light intensity of 40 μmol m<sup>-2</sup> s<sup>-1</sup>.

To avoid major statistical errors, each treatment was replicated in six culture flasks, each of them containing five microshoots. Observations were made every four weeks, at the time of shoots separation from the formed clusters and their subcultivation of fresh medium (with the same

composition). The multiplication rate was calculated from the ratio between the number of shoots regenerated per explant cultivated *in vitro* and the number of initial shoots in each subculture, for each of the culture medium used for micropropagation.

Processing of data obtained from the experiments of micropropagation was performed by analysis of variance with Duncan's Multiple-Range test, using the SPSS for Windows, version 16.0 (2007). The experimental results (mean values and percentage values) are presented and analyzed in this paper in several relevant graphics.

**Table 1. Composition of the culture medium used for *in vitro* micropropagation of shoots in 'Nero' and 'Melrom' cultivars of *A. melanocarpa* (Michx.) Elliot**

Treatment	Basal medium	Growth regulators (mg × dm <sup>-3</sup> )		
		GA <sub>3</sub>	BA	IBA
V1	Macroelements MS n, Microelements LF n, Vitamins LS n	0,1	1,00	0,1
V2	Macroelements MS n, Microelements LF n, Vitamins LS n	0,1	1,50	0,1
V3	Macroelements MS n, Microelements LF n, Vitamins LS n	0,1	1,00	0,2
V4	Macroelements MS n, Microelements LF n, Vitamins LS n	0,1	1,50	0,2

### 3. RESULTS AND DISCUSSION

**The influence of genotype and culture medium on the *in vitro* multiplication rate.** Rinsing of the buds of *Aronia melanocarpa* (Michx.) Elliot with 96% ethanol and 6% calcium hypochlorite resulted in an effective surface sterilization, with a contamination percentage of less than 12% of explants.

The calculated value of regeneration ability of shoot apices originating from mature donor plants was 86,6%. Statistical analysis of the results revealed that the potential of micropropagation by axillary shoot formation in cultivar 'Nero' was significantly influenced by the composition of the culture medium. Increased concentrations of auxin and cytokinin (V4) induced a higher rate of shoot multiplication in cultivar 'Melrom', and a significantly higher rate in cultivar 'Nero', compared to the other three treatments (Fig. 1). In this context, it should be noted that for the *in vitro* stimulation of regeneration of shoots from axillary buds of *A. melanocarpa* (Michx.) Elliot, the basal media used most often were MS, half-strength MS, and Woody Plant Medium (Brand and Cullina, 1990; Litwińczuk, 2013; Kwak *et al.*, 2015). The significant difference in the shoot proliferation ability of the two studied chokeberry cultivars can be attributed to the influence of genotype. Similar results were obtained by Kwak *et al.* (2015), who reported a higher rate of proliferation in cultivar 'Nero', compared to cultivars 'Purple', 'Mackenzie', 'Viking' and 'Odama machiko', on WPM culture medium supplemented only with 1,0 mg × dm<sup>-3</sup> zeatin. In the study carried out by us, the greater number of microshoots regenerated in cultivar 'Nero', regardless

of the composition of the culture medium, can be attributed to the growth regulators balance, defined by a substantially higher concentration of cytokinin.

**The frequency of shoot regeneration in successive subcultures.** For both ‘Nero’ and ‘Melrom’, the potential of shoot regeneration from axillary buds and proliferation during the three subcultures was increasing (Fig. 2). Regardless of the composition of the culture medium, cultivar ‘Nero’ showed a statistically significant higher frequency of shoot regeneration during the all three successive subcultures, compared to cultivar ‘Melrom’.

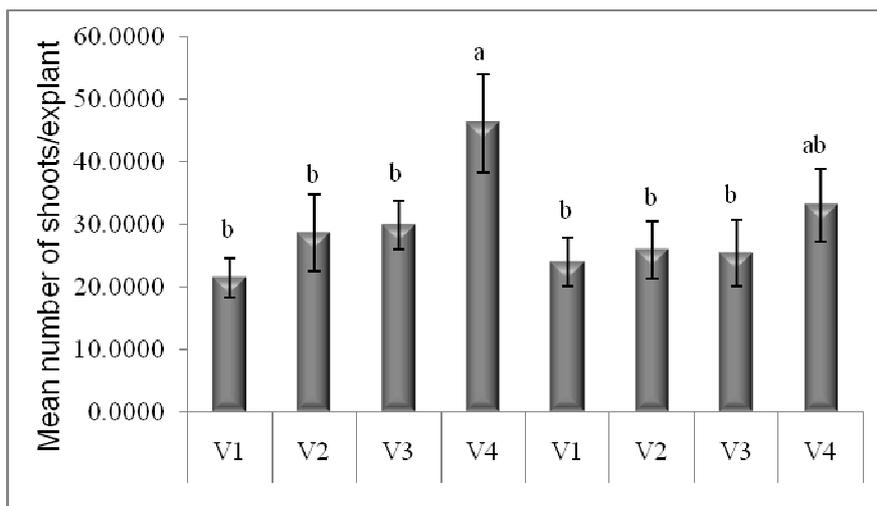


Figure 1. The influence of genotype and culture medium on the in vitro shoot multiplication rate in cultivars ‘Nero’ and ‘Melrom’ of *A. melanocarpa* (Michx.) Elliot (bars represent standard deviation of the mean; a, b: interpretation of the significance of differences by the Duncan test,  $p < 0,05$ ).

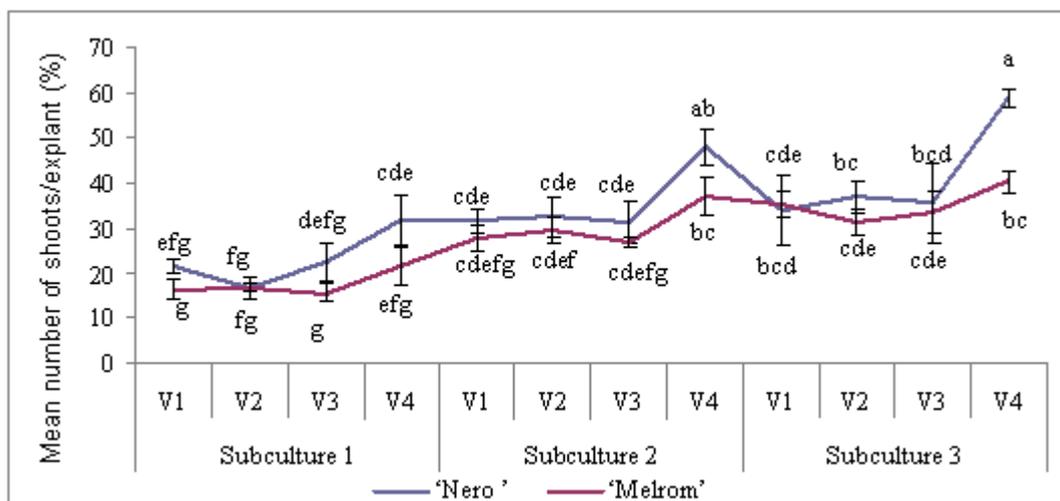
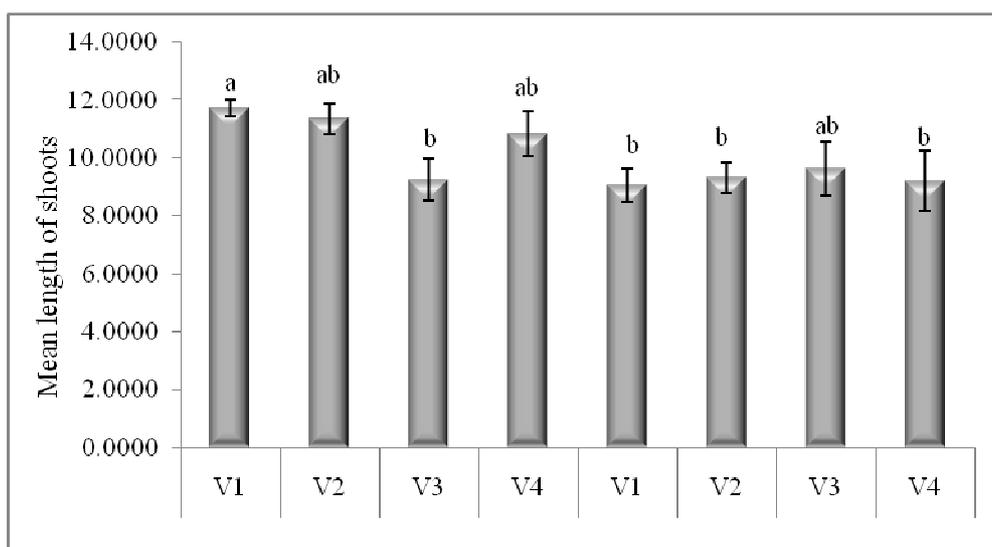


Figure 2. Shoot proliferation rate in cultivars ‘Nero’ and ‘Melrom’ of *A. melanocarpa* (Michx.) Elliot in successive subcultures (bars represent standard deviation of the mean; a, b, c, d, e, f, g: interpretation of the significance of differences by the Duncan test,  $p < 0,05$ ).

**The influence of genotype and composition of culture medium on the length of shoots.** Compared to the cultivar ‘Melrom’, shoot length was greater and significantly higher in cultivar ‘Nero’, excepting those regenerated in treatment V3, defined by a lower concentration of BA and a high concentration of IBA. However, it should be pointed out that, on average, shoots regenerated on the culture medium from V1 treatment were significantly higher, which is likely to reflect the influence of the cytokinin-auxin balance on cell proliferation. Also, the different response of the two chokeberry cultivars to the *in vitro* environment confirm the influence of genotype on the ability of cellular proliferation and regeneration *in vitro*. Actually, the studies on the micropropagation ability of various species of trees and shrubs, such as *Ribes uva-crispa* (Wainwright and Flegmann, 1986), *Rubus idaeus* (Popescu and Isac, 2000; Isac and Popescu, 2009), *Vaccinium macrocarpon* (Debnath and McRae, 2001), *Rubus chamaemorus* (Martinussen et al., 2004), *Vaccinium vitis-idaea* (Debnath, 2005), *Rubus fruticosus* and *Ribes nigrum* (Ruzic and Lazic, 2006), *Sorbus aucuparia* (Lall et al., 2006), *Arbustus unedo* (Gomes and Canhoto, 2009), *Ribes rubrum* (Sedlák and Paprštejn, 2012), *Paulownia* sp. (Shtereva et al., 2014) *Prunus dulcis* (Choudhary et al., 2015), and many other species, such as *Fragaria* and *Potentilla* (Isac et al., 1994; Şuţan et al., 2010), revealed the influence (from slight to strong) of genotype.



**Figure 3.** The influence of genotype and composition of culture medium on the length of shoots in cultivars ‘Nero’ and ‘Melrom’ of *A. melanocarpa* (Michx.) Elliot (bars represent standard deviation of the mean; a, b: interpretation of the significance of differences by the Duncan test,  $p < 0,05$ )

As shown in figure 4, in both chokeberry cultivars studied, the cell proliferation was not significantly influenced by the number of subcultures.

In both ‘Nero’ and ‘Melrom’, the analysis of correlation between the number of shoots and the shoot length, by calculating the Pearson’s correlation coefficient, showed a very weak inverse correlation between these two parameters, with  $r = -0,091$  in cultivar ‘Melrom’, and  $r = -0,098$  in cultivar ‘Nero’, respectively.

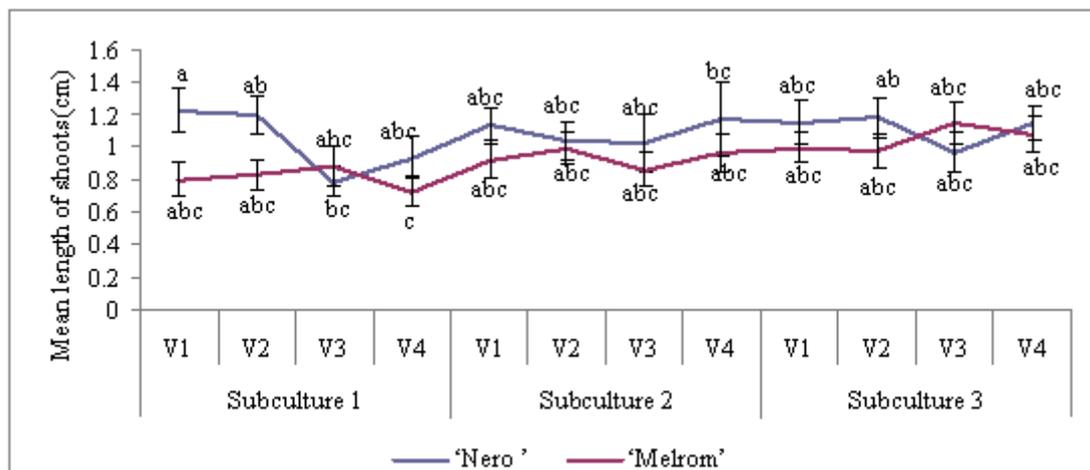


Figure 4. The influence of the number of subcultures on the length of shoots (bars represent standard deviation of the mean; a, b, c: interpretation of the significance of differences by the Duncan test,  $p < 0,05$ )

#### 4. CONCLUSIONS

The influence of genotype proved to be extremely important in the *in vitro* micropropagation of chokeberry cultivars 'Nero' and 'Melrom', revealing the higher potential of cell proliferation and shoot regeneration of 'Nero'. Regardless of the genotype and culture medium, the *in vitro* shoot multiplication rate was increasing during the first three subcultures. The overall results of our study showed also that the ratio of auxin, cytokinin and gibberelic acids important for maintaining a proper growth of shoots in length during the successive subcultures.

#### 5. ACKNOWLEDGEMENTS

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