Scientific Papers. Series B, Horticulture. Vol. LXIII, No. 1, 2019
Print ISSN 2285-5653, CD-ROM ISSN 2285-5661, Online ISSN 2286-1580, ISSN-L 2285-5653

HIGH EFFICIENCY SHOOT MULTIPLICATION FROM *IN VITRO* CULTURED MERISTEMS OF *ARONIA MELANOCARPA* CV. NERO

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Abstract

Axillary shoot meristems of black chokeberry (Aronia melanocarpa) cv. 'Nero', with 1-2 leaf primordia, were aseptically isolated and cultured on solid Murashige and Skoog (MS) medium supplemented with N^6-benzyladenine (BA, 0.5 - 2.0 mg/l) and indole-3-butyric acid (IBA, 0.1 - 1.0 mg/l). Although the frequency of shoot formation from in vitro cultured meristems was over 70% in all the four treatments, primary shoot induction was most effectively promoted by MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l IBA. In this combination all the excised meristems responded by developing vigorous shoots, with some slight callus formation. Following establishment, based on the results of several preliminary testing experiments, the primary shoots were further cultured on solid MS medium supplemented with 1.0 mg/l BA + 0.1 mg/l IBA for proper shoot multiplication. The number of shoots formed per initial shoot varied largely (from 2 to 31), with an average multiplication rate of 7.84, and their length was found to be strongly dependent on their number. Thus, the length (revealing the vigour) was higher for shoots formed in lower number per initial shoot, reaching up to 3.3 cm after four weeks of in vitro culture. Almost all the developed shoots were vigorous enough for further multiplication by subculturing them on fresh MS medium every four weeks. This protocol would be useful for large scale micropropagation of black chokeberry cultivars from meristem explants.

Key words*: black chokeberry, in vitro, meristem-derived plantlets, growth regulators, shoot multiplication, multiplication rate.*

INTRODUCTION

Aronia melanocarpa (Michx.) Elliot (black chokeberry), is a shrub highly appreciated for its edible berries, which constitute a very rich source of numerous substances exerting a beneficial impact on health (McKay, 2001, Tanaka and Tanaka, 2001; Valcheva-Kuzmanova and Belcheva., 2006, Jakobek *et al*., 2007; Walther and Schnell, 2009; Rop *et al*., 2010; Jakobek *et al*., 2012; Litwinczuk, 2013). 'Nero', 'Viking', 'Rubina', 'Purple, 'Mackenzie' 'Galicjancka', 'Odamamachiko' 'Hugin', 'Aron', 'Fertödi' and 'Melrom' are among the most known and popular varieties of *A. melanocarpa* in Europe (Strigl *et al*., 1995, Kulling and Rawel, 2008; Walther and Schnell, 2009; Kwak *et al*., 2015; Borowska and Brzoska, 2016; Rusea *et al*., 2018). They differ from each other by the weight and diameter of the fruit, efficiency of juice extraction, content of total polyphenols, anthocyanins and proanthocyanidins, as well as the total antioxidative capacity, (Rop *et al*., 2010; Ochmian *et al*., 2012; Rugină *et al*., 2012).

The polyphenols from *Aronia melanocarpa* fruit juice (mainly proanthocyanidins, anthocyanins, flavonoids, and phenolic acids), have been demonstrated to possess antioxidative, anti-inflammatory, antiviral, anticancer, anti-atherosclerotic, hypotensive, antiplatelet, and antidiabetic properties (Benvenuti *et al*., 2004; Slimestad *et al*., 2005; Naruszewicz *et al*., 2007; Denev *et al*., 2012; Jakobek *et al*., 2012; Bădescu *et al*., 2015; Borowska and Brzóska, 2016; Park *et al*., 2017). It is currently considered that black chokeberry possesses one of the highest antioxidant activities among fruits. *A. melanocarpa* berries are also known to be rich of cyanidin glycosides (Wiczkowski *et al*., 2010).

From the researches on the antioxidant (Kahkonen *et al*., 2001; Wu *et al*., 2004; Oszmianski and Wojdylo, 2005; Olas *et al*., 2008; Denev *et al*., 2012), anti-inflammatory (Zapolska-Downar *et al*., 2012), hepatoprotective (Kowalczyk *et al*., 2003; Valcheva-Kuzmanova *et al*., 2004), cardioprotective (Naruszewicz *et al*., 2007), hypotensive and lipid lowering (Hellstrom *et al*., 2010; Park and Park 2011), hypoglycaemic and antidiabetic effects (Simeonov *et al*., 2002; Rugină *et al*., 2011; Bădescu *et al*., 2015; Banjari *et al*., 2017), to those on antimutagenic (Gasiorowski *et al*., 1997; Duthie *et al*., 2007) and antitumoral effects (Malik *et al*., 2003; Bermudez-Soto *et al*., 2007; Olas *et al*., 2010; Sharif *et al*., 2013), or those on protective action against degenerative diseases, the scientific literature is rich in information highlighting their prophylactic and therapeutic properties, without suggests on any unwanted or side effect of their use (Kokotkiewicz *et al*., 2010).

Although micropropagation of adventitious shoots formed through organogenesis from somatic tissue explants could be a suitable alternative in *Aronia melanocarpa* (Rusea et al., 2018), multiplication of shoots formed from axillary buds is seen as the most applicable and reliable method of *in vitro* propagation (Şuțan et al., 2017).

Successful plant propagation from *in vitro* cultured meristems has been reported for *Aronia* species, including *A. melanocarpa* (Brand and Cullina, 1990; Brand and Cullina, 1992; Petrović and Jacimović-Plavsić, 1992; Velchev and Mladenova, 1992; Staniene *et al*., 1999; Litwińczuk, 2002; Mahečić, 2009; Litwinczuk, 2013; Kwak *et al.,* 2015; Şuțan *et al*., 2017) and *A. arbutifolia* (Kane *et al*., 1991).

It is well known that shoot multiplication is highly dependent on basal medium composition, and type and concentrations of plant growth regulators (Popescu and Isac, 2000; Isac and Popescu, 2009; Atak and Çelik, 2012; Křižan *et al*., 2013). However, the multiplication rate (index) in different genotypes (cultivars) cultured *in vitro* on a certain medium might be significantly different (Popescu and Isac, 2000).

Little is known about the multiplication rate of *Aronia melanocarpa* genotypes in succesive subcultures. Generally, subculture effect on multiplication rate of *in vitro* cultures varies from one species to another (Vujović *et al*., 2012). In some woody plant species, the shoot multiplication index increased with subculturing. Thus, in dwarf raspberry (*Rubus pubescens* Raf.), Debnath (2004) noticed that shoot multiplication index, as well as shoot length and leaf number, increased with subculturing up to the third subculture period, and then remained constant. Similarly, the increase in shoot production with extended time of culturing was reported in cherry and apple (Grant and Hammat, 1999).

Nevertheless, a decrease in multiplication potential during repeated subculturing of shoots on medium of constant hormonal composition was reported in some species and cultivars of *Rosaceae* (Norton and Norton, 1986; Vujović *et al*., 2012; Aygun and Dumanoglu, 2015). The point of decline is highly dependent on treatments applied (hormonal composition of medium, incubation period, etc).

In the present study, we investigated the efficiency of shoot multiplication from *in vitro* cultured meristems of 'Nero', one of the most valuable black chokeberry cultivars in both central and south-eastern Europe.

MATERIALS AND METHODS

The meristems from 'Nero' cultivar of *Aronia melanocarpa* (Michx.) Elliot were excised from axillary buds of field-grown plants in the small fruits collection of the Research Institute for Fruit Growing, Piteşti.

In vitro culture initiation. Branches of cultivar 'Nero' were harvested in the middle of spring from the field-grown plants and pre-sterilized by washing in tap water to which 2-3 drops of Domestos were added. Subsequently, axillary buds were disinfected successively with 96% ethanol for 5 minutes and with 6% calcium hypochlorite for 10 minutes. After sterilization, the biological material was rinsed in three baths of distilled water.

Meristems 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 glass tubes of 20-25 cm^3, containing 10 ml of culture medium sterilized by autoclaving.

The culture medium containing Murashige-Skoog (1962) macroelements, microelements and vitamins, 20 g × dm^{-3} sucrose, 0,1 mg × dm^{-3} giberellic acid (GA_3) and 1 mg × dm^{-3} benzyladenine (BA) was solidified with 7 g/l agar.
The pH of the culture medium was adjusted to 5.7 with 0.1 N KOH before autoclaving for 20 minutes at 121 ^{0}C.

Multiplication of meristem-derived plantlets.
Meristem-derived plantlets were divided and transferred into Ehrlenmayer flasks of 100 cm^3 capacity, containing 30 ml of culture medium composed of Murashige-Skoog (MS) macro-elements, microelements and vitamins, and combinations of BA and IBA (Table 1).
For culture media preparation, separate stock solutions of macroelements and micro-elements were used. Iron was added to the medium as separate stock solution of ferric sodium salt EDTA (32 mg L^{-1}). BA and IBA were disolved in 1N HCl and 1N NaOH, respectively. Dextrose was used as carbon source in the culture media (40 g L^{-1}). In all experiments the culture media were solidified with 8 g × dm^{-3} agar.
The pH of the culture medium was adjusted to 5.7 with 0.1 N KOH before autoclaving for 20 minutes at 121 ^{0}C.
The culture flasks were sealed with plugs of cotton wrapped in aluminium foil and then autoclaved.
The *in vitro* cultures of meristem-derived plantlets 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^{-2} s^{-1}.

Shoot multiplication
Regardless of the experimental variant of culture medium on which they formed, the shoots multiplied from the meristem-derived plantlets were transferred in glass jars of 350 ml on the medium which gave the best multiplication index. The transfer was carried out when the shoots reached a length of 1-2 centimeters.
The *in vitro* cultures of shoots were incubated, identically to the meristem-derived plantlets, in growth chamber at 22-24°C, under a photo-period of 16 hours light / 8 hours darkness, and a light intensity of 40 µmol m^{-2} s^{-1}.
In order to correctly interpret the results of multiplication in the first and second subcultures and to avoid major statistical errors, four shoots were placed in each glass jar, in at least six repetitions.
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 formed per explant (single shoot) cultivated *in vitro* and the number of initial shoots in each subculture on the culture medium used for multiplication.
Data for both multiplication of meristem-derived plantlets and subsequent shoot multiplication in the first and second subculture were analyzed for significance by the standard analysis of variance with mean separation by Duncan's test ($p > 0.05$).

Table 1. Composition of the culture media used for *in vitro* multiplication of meristem-derived plantlets in 'Nero' cultivar of *A. melanocarpa* (Michx.) Elliot

Experimental variant	Basal medium	Growth regulators (mg L^{-1})	
		BA	IBA
V1	MS	0.5	0.1
V2	MS	1.0	0.1
V3	MS	2.0	0.5
V4	MS	2.0	1.0

RESULTS AND DISCUSSIONS

The meristem-derived plantlets of black chokeberry cultivar 'Nero' developed tiny shoots in four to six weeks on all the four MS culture media with different combinations of benzyladenine (BA) and 3-indolyl-butyric acid (IBA). As shown in Table 2, the highest

number of shoots was formed by meristem derived-plantlets cultured on MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l IBA (an average of 10.2 shoots per meristem-derived plantlet), followed closely by those cultured on MS medium supplemented with 0.5 mg/l BA and 0.1 mg/l IBA (an average of 9.67 shoots per meristem-derived plantlet).

Although the average number of shoots formed by meristem derived-plantlets cultured on MS media supplemented with 1.0 mg/l BA and 0.1 mg/l IBA and, respectively, 2.0 mg/l BA and 1.0 mg/l IBA was only 6.68 and 6.08 respectively, no significant differences were found between the four experimental variants when analyzed by Duncan's test (Table 2).

However, significant differences were found in the length of shoots formed by meristem-derived plantlets cultured on MS medium with different concentrations of growth regulators. Thus, after six weeks in culture, the highest average length of shoots (2.24 cm) was found in the experimental variant containing 2.0 mg/l BA and 0.5 mg/l IBA (Table 2), the same which gave the highest average number of shoots per meristem-derived plantlet. The Duncan's test showed that in this experimental variant of culture medium, and also in that containing 1.0 mg/l BA and 0.1 mg/l IBA, the average length of newly formed shoots (Figure 2 and Figure 3) is significantly higher then in the other two experimental variants (Table 2).

The combination of growth regulators (2.0 mg/l BA and 0.5 mg/l IBA) added to the MS medium for the multiplication of cultivar 'Nero' single shoots in subsequent subcultures allowed high rates of multiplication (Table 3). The best rate of shoot multiplication was achieved in the second subculture, with 25 newly formed shoots per single shoot. Moreover, as shown by the Duncan's test, the multiplication rate (index) in the second subculture was significantly higher than that in the first subculture. These results are consistent with those reported in other plant species by Grant and Hammat (1999), Paudyal and Haq (2000), Debnath (2004), and others.

Table 2. Average number of shoots formed by meristem-derived plantlets of cultivar 'Nero' cultured *in vitro* on MS basal medium supplemented with combinations of BA and IBA

Experimental variants	Number of meristem-derived plantlets	Number of shoots / meristem-derived plantlet	Shoot length (cm)
V1 (MS + 0.5 mg/l BA + 0.1 mg/l IBA)	14	9.67 ± 1.57 a	1.72 ± 0.12 bc
V2 (MS + 1.0 mg/l BA + 0.1 mg/l IBA)	15	6.68 ± 1.01 a	1.95 ± 0.07 ab
V3 (MS + 2.0 mg/l BA + 0.5 mg/l IBA)	14	10.2 ± 1.81 a	2.24 ± 0.14 a
V4 (MS + 2.0 mg/l BA + 1.0 mg/l IBA)	13	6.08 ± 0.55 a	1.45 ± 0.05 c

* Values presented are mean ± SE. Means followed by the same letter are not significantly different (Duncan test, $p>0.05$)

The statistical interpretation of data showed also that, despite of the significantly higher number of shoots formed from each single shoot in the second subculture, there were no significant diferences in the vigor of newly produced black chokeberry shoots in the second subculture, compared to the first subculture (Table 3). However, the shoot length varied during subculturing, with the highest values of this parameter observed in the second subculture.

Hamad and Taha (2008) reported that the subcultures improved shoot elongation. In contrast, Norton and Norton (1986) reported a decrease in shoot length and leaf size after several in vitro subcultures. Therefore, further observations needs to be made in subsequent subcultures for the multiplication of black chokeberry cultivar Nero.

No visible morphological variations or aberrations of shoots were found in the first and second subcultures (Figure 3 and 4).

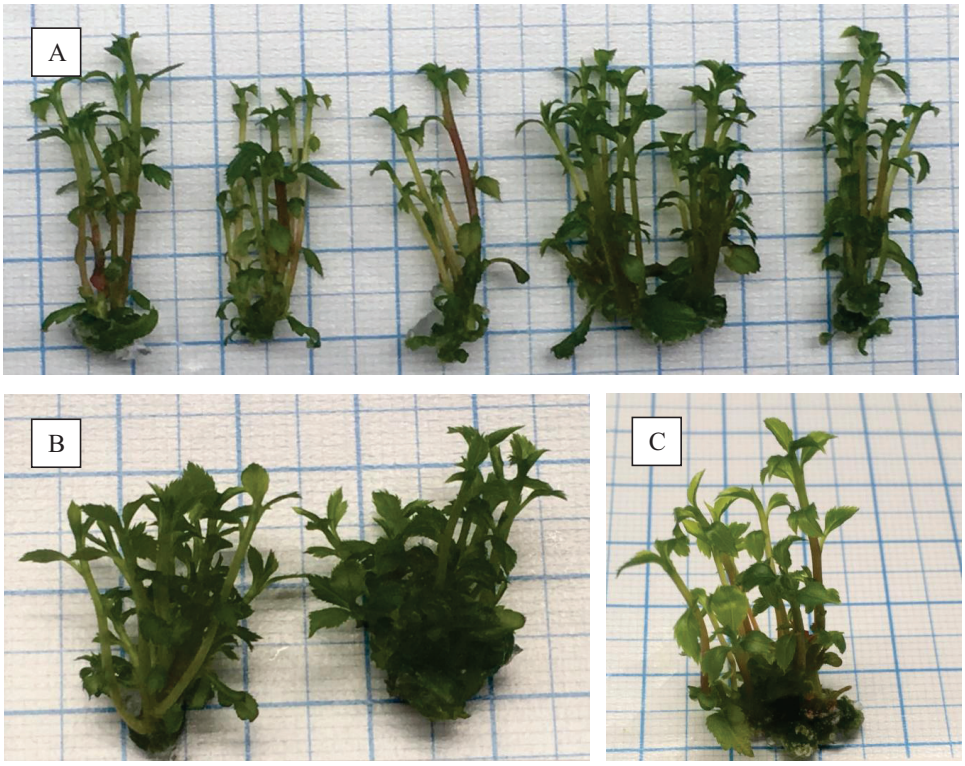

Figure 1. A-C: Multiplication of shoots formed from meristem-derived plantlets of black chokeberry cultivar ‘Nero’.

Table 3. Average number of shoots formed per single shoot of cultivar ‘Nero’ in the first and second subculture, respectively, on MS medium supplemented with 2.0 mg/l BA + 0.5 mg/l IBA.

Subculture	Number of shoots / plantlet (single shoot)	Shoot length (cm)
1st	7.83 ± 0.62 b	1.74 ± 0.17 a
2nd	25.0 ± 3.99 a	2.24 ± 0.31 a

* Values presented are mean ± SE. Means followed by the same letter are not significantly different (Duncan test, p>0.05).

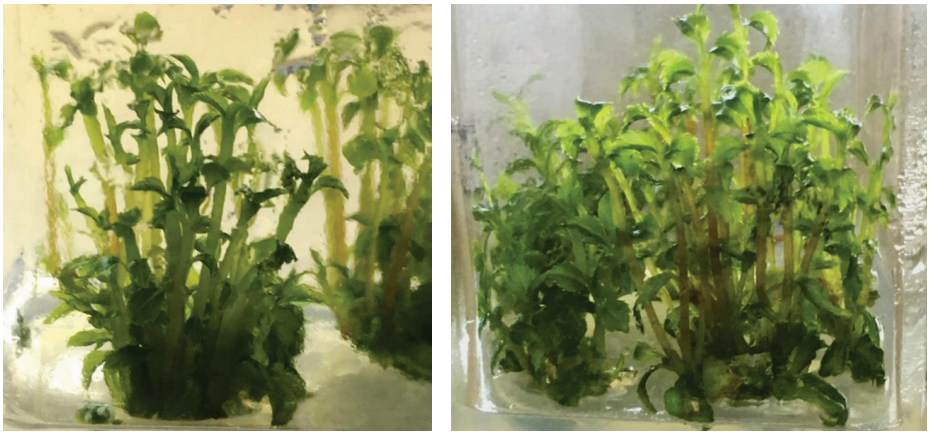

Figure 2. High rate of shoot multiplication in the first (left) and second subculture (right), respectively, on Murashige-Skoog medium supplemented with 2.0 mg/l BA + 0.5 mg/l IBA.

Figure 3. A and B: Length of shoots multiplied from single shoots separated from clusters formed by meristem-derived plantlets of chokeberry cultivar 'Nero', after six weeks of *in vitro* culture

In some previous experiments, where plantlets developed from axillary or apical meristems of black chokeberry were used as initial explants, higher shoot multiplication rates than in present experiments were found in the first two subcultures. For instance, with cultivar 'Nero', Borsai *et al*. (2017) reported a rate of shoot multiplication of 49. However, the number of shoots produced per subculture strongly decreased during further subcultures.

The effectiveness of MS medium for *in vitro* culture of chokeberry was emphasized in many published reports (Brand and Cullina, 1990; Kane *et al*., 1991; Brand and Cullina, 1992; Petrovic and Jacimovic-Plavsic, 1992; Velchev and Mladenova, 1992; Staniene *et al*., 1999; Mahečić, 2009; Litwinczuk, 2013; Șuțan *et al*., 2017).

Although the use of Woody Plant Medium (WPM) (Lloyd and McCown, 1980) became frequent with woody plants, including *Aronia* (Kwak *et al*., 2015; Borsai ei al., 2017; Chen, 2017), MS medium is a choice with reliable results, supported by many authors, such as Brand and Cullina (1990; 1992), who reported that both MS medium and WPM medium supported vigorous shoot proliferation in *Aronia arbutifolia* and *A. melanocarpa*.

In experiments carried out by Borsai *et al*. (2017) WPM, supplemented with either 2 mg L-1 zeatin or 5 mg L-1 2-iP provided the lowest multiplication rates (17.5 and 13). The same authors reported that the shoot number and shoot length in MS medium proved to be superior compared to the other media tested, such as Driver Kunyiuki Walnut (DKW) and Woody Plant Medium (WPM).

In our experiments with cultivar 'Nero' of black chokeberry, the MS medium was proven to be very suitable for a high multiplication rate

of meristem-derived plantlets and also of single shoots separated from clusters, in the subsequent two subcultures. Thus, our results are consistent with those reported by Mahečić (2009), Borsai *et al*. (2012), and Litwinczuk (2013).

Although a high frequency of shoot formation is most often desired, clusters with less then twenty shoots are generally advantageous, because they have a higher vigor (and consequently a better ability to multiplicate), and also because the thin and crowded clusters of shoots could not be separated easily for the stage of multiplication.

Moreover, a high rate of multiplication is associated in most plant species with low ability of shoots to elongate, and generally with low percentages of newly formed shoots reaching the length and vigor needed to be subcultured without a drastic decline of ability to multiplicate during the next subculture. Otherwise, in order to assure a high efficiency of multiplication, the tiny, low vigor shoots from large clusters should be maintained after the separation of well developed shoots, and cultured further on fresh medium untill they reach the parameters suitable for their use as single shoots for subsequent multiplication.

CONCLUSIONS

The results of our studies aiming at investigation of the efficiency of *in vitro* shoot multiplication from meristem-derived plantlets of black chokeberry cv. 'Nero' showed a high rate of multiplication in both the first and second subcultures, with a significant increase at the later.

Our results showed the possibility of *in vitro* multiplication at high rate of shoots developed by meristem-derived plantlets, which holds great promise for rapid and efficient micro-propagation of black chokeberry cultivar Nero, and probably of some other *Aronia melanocarpa* cultivars and elite genotypes.

ACKNOWLEDGEMENTS

This research was supported by a Doctoral Research Support grant from the Ministry of National Education. We greatly appreciate the support received through the collaborative work undertaken with the Laboratory of Tissue Culture at the Research Institute for Fruit Growing, and University of Pitești.

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