IN VITRO PROPAGATION OF *VACCINIUM MYRTILLUS* L.

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Abstract

The study aims to support a method of initiation in vitro cultures of seeds and minicuttings of bilberry (Vaccinium myrtillus) and to identify the effect of activated charcoal on Vaccinium myrtillus inoculums in subculture, to optimize rootedness or callus process, even in the absence of growth regulators. Initiation of in vitro cultures of bilberry (Vaccinium myrtillus) was successful, either from seeds or from minicuttings, on medium MB-MS - G with the addition of 2 mg/ l BA and 0.1 mg / l IBA. Activated charcoal 2% added to the in vitro bilberry culture medium led to stimulate only caulogenesis, but to decrease the survival rate of seedlings, by changing the pH of culture medium, which increases the value of 8, which favoured - in our laboratory conditions - the emergence of infections loving alkaline pH and loss of culture. In the step of multiplication of the callus, the activated charcoal led to lower its rate of necrosis, but also to changes in pH, as well. The initiation and proliferation of callus from bilberry was carried out under optimum conditions on culture medium with the addition of 3 mg /l 2.4 D, lacking activated charcoal, to yield a friable callus.

Key words: *Vaccinium*, micropropagation, activated charcoal, seeds, minicuttings, callus

INTRODUCTION

The bilberry (*Vaccinium myrtillus L.*) is a species which is part of Magnoliopsida Class, Ericales Order, Ericaceae Family, Gen *Vaccinium* (Săvulescu, 2007). The varieties of bilberries are usually propagated by cuttings sized from the stem, in order to maintain their true morphological and genetic structure. Lately were developed methods of mass propagation using cuttings from bilberry (Meiners et al., 2007), but some clear disadvantages can be optimized.

Conventional propagation methods are time consuming, require large amounts of starting material and large spaces for incubation, involve less control regarding pathogen material and deficiencies in formation of the root system to certain varieties. It is also difficult to obtain homogeneous descendants (Ostrolucká et al., 2004). The performance reached by *in vitro* bilberry plants and two years in the natural medium are larger than those obtained by traditional cultures. So they grow more uniform and have no morphologic variability (Ostrolucká et al., 2007). Instead, Litwińczuk et al. (2005) stated that weren`t differences between the bilberry plants obtained *in vitro* from axillary and adventitious buds and the traditional cultivation; however there were features significantly diminished when were used materials derived from *in vitro* culture for 11 years, leading to the

conclusion that are necessary frequent culture units (every two or three years) to overcome these effects.

Research on *in vitro* cultivation at genus *Vaccinium* were and are of great interest, internationally there are numerous reports in connection with attempts of optimize procedures, either from perspective of culture medium optimization (Reed, 1991; Rowland, Ogden, 1992; Cao et al., 2003; Debnath, 2007; Ostrolucká et al., 2009; Kudryashova et al., 2012), or in that of the use of advanced types of explants, such as meristems or leaves (Ostrolucká et al., 2004, 2007; Gajdošová et al., 2006; Fira et al., 2008; Debnath, 2009; Zhao et al., 2011; Vescan et al., 2012).

Although micropropagation automatization in bioreactors developed as a possible way to reduce the cost of propagation, optimum production of plants depends on a better understanding of the physiological and biochemical responses of plants at micromedium culture signals an optimization of specific condition of physical and chemical culture to control plant morphogenesis of wild berries in liquid culture systems (Debnath et al., 2012).

Because traditional bilberry propagation methods have several disadvantages, the purpose of this study was to identify new procedures applied *in vitro* to achieve a micropropagation protocol at this species.

MATERIAL AND METHOD

The vegetal material consisted, either from seeds or binodal cuttings from *Vaccinium myrtillus L.*. The experimental protocol is described in Table 1. Research was done between June 2015 - April 2016.

RESULTS AND DISCUSSION

Step I. Following placement of the seeds on sterile culture medium, the germination process starts at 14 days after inoculation (Fig. 1 and Fig. 2), with a smaller germination percentage value, compared to total number of seeds provided to germinate, but the germination percentage increased to 78% after 30 days from the initiation of culture (Fig. 2), and at 60 days, was recorded maximum of germinated seeds, of 80% (Fig. 2).

In terms of growth indices, at 90 days after seeds placement on sterile medium have been identified plantlets with an embryonic root average length of 0.4 cm, with strains average length of 1.6 cm, approx. 1-2 per plantlet. In some nodes leaflets being fallen and signalled the presence of an average number of leaflets, slightly low - only to the number of nodes (Fig. 3) (at bilberry at each node is located a leaflet, and these are arranged alternate on the stem). The average size of the whole plant, obtained by

adding the length of the root to that of the stem, was in average of 2 cm (Fig. 3).

Table 1

Research design

Step I –*in vitro culture initiation*		
Explant type	a) Seeds	b) binodal minicuttings
Sterilization	Alcohol 70% - few seconds submersion Sodium hypochlorite 2% + Tween 80 - 15 minutes Clean with sterile water – 25 minutes	
Culture medium	Basal, solidified MB-MS+G, with 0,1 mg/l IBA and 2 mg/l BA	
Culture recipients	Uncoloured glass recipients, with 11 cm height and 2 cm diameter	
In vitro growth conditions	1700 lx, 16/24 h light, 24 – 25 ˚C	
Culture period	90 days	30 days
Step II – a) subculture		
Inoculs type	binodal minicuttings	
Culture medium	V_0 - MB-MS+G, with activated charcoal 2%, **without** growth regulators (*control*)	V_1- MB-MS+G, with activated charcoal 2% and **with** 0,1 mg/l IBA and 2 mg/l BA
Culture recipients	Uncoloured glass recipients, 11/2 cm	
In vitro growth conditions	1700 lx, 16/24 h light, 24 – 25 ˚C	
Culture period	60 days	
Step II – b) callus initiation and callus subculture		
Inoculs type	binodal minicuttings	
Culture medium for callus initiation	Basal, solidified MB-MS+G, with 3 mg/l 2,4 D	
Culture recipients	Uncoloured glass recipients, 11/2 cm	
In vitro growth conditions	1700 lx, 16/24 h light, 24 – 25 ˚C	
Culture period (1)	30 days	
Medium for subculture	Basal, solidified MB-MS+G, with 3 mg/l 2,4 D **with** and **without** activated charcoal 2%	
Culture recipients	Uncoloured glass recipients, 11/2 cm	
In vitro growth conditions	1700 lx, 16/24 h light, 24 – 25 ˚C	
Subculture period (2)	60 days	

Note: MB-MS – basal Murashige-Skoog (1962) medium; G – Gamborg . (1968) vitamins; IBA – indole-3-butiric acid; BA – n^6-benzyladenine.; 2,4 d - dichlorophenoxyacetic acid

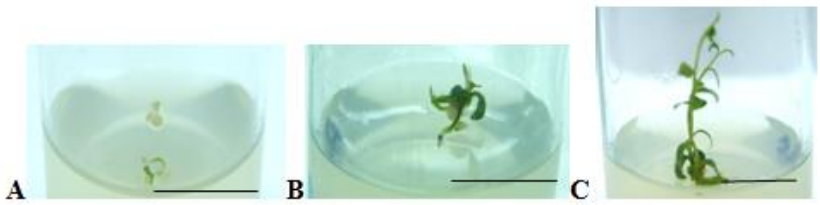

Fig. 1. Aspects of bilberry seedlings (*Vaccinium mytillus*), on 14 (A), 30 (B) and 60 (C) days after placing the seeds on septic medium (bar means 1 cm)

Debnath (2007) studied the effects of four concentrations of indole-3-butyric acid (IBA) and two methods of propagating at the bilberry (*Vaccinium angustifolium Ait*.). The author noted that IBA had an effect on the morphology of propagated plants, increasing the concentration of IBA led to stem and leaf growth on the stem. Stems have grown with IBA concentrations up to 20 μM.

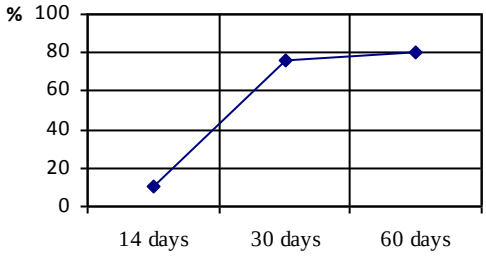

Fig. 2. Evolution of bilberry germination process (*Vaccinium myrtillus*), using seeds placed on sterile medium

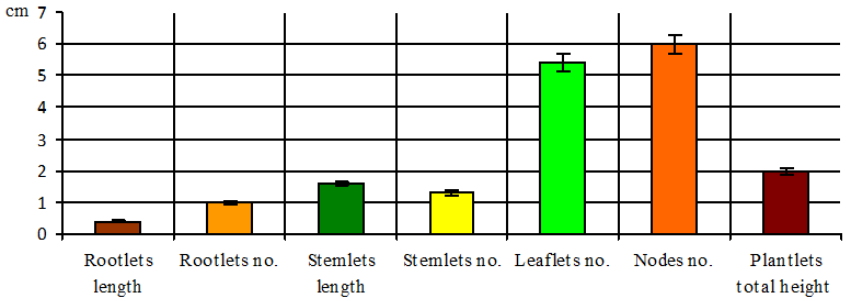

Fig. 3. Average growth indexes registered on plants of bilberry (*Vaccinium myrtillus*), from seeds to 90 days after initiation *in vitro* culture (bars mean standard deviation)

In the case of initiation of *in vitro* cultures from binodal mini cuttings, where the first node from basal pole was ½ placed in culture medium and the second node was 100% within the air space of the container, neoplantlets regeneration has occurred in the upper node (Fig. 4), in the lateral meristems. At 30 days from start up the initiation of mini cuttings, neoplantlets formed *in vitro* possessed 3-4 nodes, 3-4 leaf and a stem length of 0.7 cm on the average (Fig. 4).

Fig. 4. Neoplantlet of bilberry (*Vaccinium myrtillus*) regenerated at the level of minicutting node, at 30 days after placing on sterile medium

At 60 day of subculture on medium with addition of activated charcoal, with or without growth regulators (Fig. 5 and Fig. 6) has revealed an increase in waist of plantlets, similar, but the plantlets placed on medium with hormonal balance by IBA and BA has indicated the presence of an abundant aerial system, due to regeneration of a large number of stems from the initial inoculum.

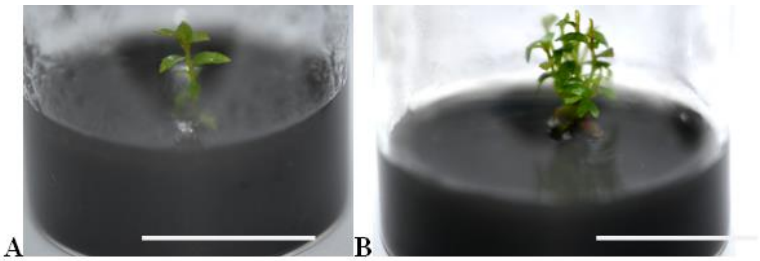

Fig. 5. Aspects of bilberry plantlets (*Vaccinium myrtillus*), regenerated from minicuttings, placed on medium V0- MB-MS+G, plus activated charcoal 2%, without growth regulators (control) (A), and V1- MB-MS+G, plus activated charcoal 2% with addition of 2 mg/l BA and 0.1 mg/l IBA (B), at 60 days of *in vitro* subculture (bar means 1 cm)

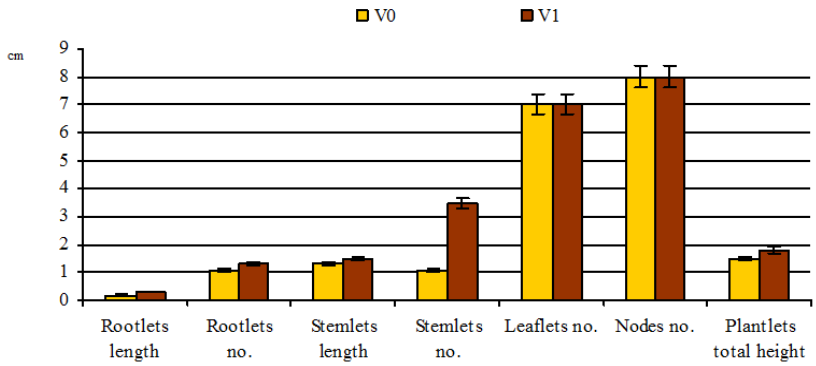

Fig. 6. Growth average indexes registered on bilberry plantlets (*Vaccinium myrtillus*), regenerated from minicuttings, at 60 day of subculture on medium V0- MB-MS+G, plus activated charcoal 2%, without growth regulators (control) (A), and V1- MB-MS+G, plus activated charcoal 2% with addition of 2 mg/l BA and 0.1mg/l IBA (B) (bars mean standard deviation)

The activated charcoal was found to be a factor that substantially modifies the pH of the culture medium, it reached in this case at the value 8. The bilberry is a species that prefers slightly acidic pH. Moreover, 1 month after the inoculation, a pH so alkaline favorized the development of alkalofile microorganisms, on the surface of the culture medium, leading to the loss of culture in proportion of 40-50% (Fig. 7). We note that this has occurred only in our laboratories, not being reported in the speciality literature and may be a local issue of pathogeny.

We have identified studies in speciality literature that confirm that sometimes the presence of activated charcoal in the culture medium may alter the pH (Owen et al., 1991). Stasinopoulos and Hangarter (1990) affirmed that may occurred photochemical changes in culture medium. Thus, the pH can be influenced by the temperature of the growth chamber, maintaining at dark or at light. And due to different absorption at phytoinoculs level, of anions and cations from the culture medium, the pH thereof is changed (George et al., 2008).

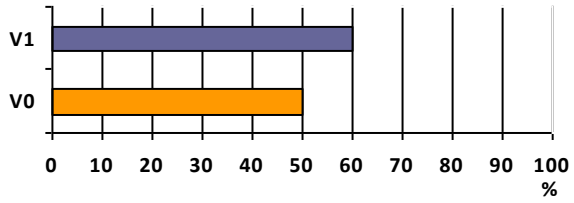

Fig. 7. The survival of bilberry (*Vaccinium myrtillus*) inoculs placed on medium V0 - MB-MS+G, plus activated charcoal 2%, without growth regulators (control) (A) and V1- MB-MS+G, plus activated charcoal 2%, with the addition of 2 mg/l BA and 0.1 mg/l IBA (B), at 30 days of *in vitro* subculture

Step II. The plantlets obtained *in vitro* from binodal minicuttings in Step I, at 30 days after the initiation were subcultured on medium with different growth regulators. These were dimensioned to uninodal minicuttings, which has been transferred in secondary culture. If regulators from subculture medium consisted on IBA 0.1 mg/l and BA 2 mg/l, were regenerated plantlets and if the growth regulator added was 2.4 D 3mg/l, from inoculs was regenerated callus. At 30 days of *in vitro* culture callus was obtained with average size of 0.7 cm, slightly depigmented. For the purpose of multiplication was realized subculturing on identic culture medium, with the addition all of the 2.4 D 3mg/l. After another 60 days of culture, callus placed on medium with 2.4 D, without activated charcoal shows on average 1.2 cm in diameter, have a yellowish green colour, was slightly friable (Fig. 8 A), with approx. 10% necrosis, being capable to be subcultured on specific medium for inducing somatic embryogenesis.

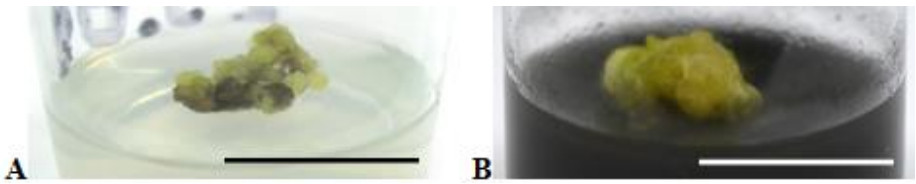

Fig. 8. Aspects of bilberry (*Vaccinium myrtillus*) callus at 60 days of subculture on medium with the addition of 2.4 D 3 mg/l, without activated charcoal (A) and with the addition of activated charcoal (B) (bar represents 1 cm)

On the other hand, at the similar variant with the addition of activated charcoal 2% was generated callus without necrosis, friable, yellowish (Fig. 8 B), but with many loss of vegetal material, from multiple infections caused by the presence of activated charcoal and the increasing of the pH of this medium, after one month from inoculation, at value of 8.

CONCLUSIONS

1. Initiation of *in vitro* cultures of bilberry (*Vaccinium myrtillus*) can be achieved successfully, either from seeds or from minicuttings, on medium MB-MS - G with the addition of 2 mg/l BA and 0.1 mg/l IBA.
2. Activated charcoal added to the *in vitro* culture medium at bilberry has led to stimulation of caulogenesis, but to decrease in survival rate.
3. Initiation and multiplication of callus at bilberry was realized in optimum condition on culture medium with the addition of 3 mg/l 2.4 D, obtaining a friable callus.
4. The activated charcoal from the culture medium used for callus multiplication, led to lower its rate necrosis.

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