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Aspects Regarding the *In Vitro* Culture and *Ex Vitro* Rooting in *Vaccinium macrocarpon* Cultivar 'Pilgrim'

Doina CLAPA[1)], Alexandru FIRA[1)], Liviu A. VESCAN[2)]

[1)]In Vitro Culture Laboratory, Fruit Research Station,
5 Horticultorilor Street, Cluj-Napoca, Romania; firaalexandru@yahoo.com
[2)]University Of Agricultural Sciences and Veterinary Medicine, 3-5 Mănăştur Street,
400372 Cluj-Napoca, Romania

Abstract. Our paper presents aspects regarding *in vitro* culture initiation in cranberry (*Vaccinium macrocarpon*), cultivar `Pilgrim`, *in vitro* multiplication and *ex vitro* acclimation. In order to establish the optimal explant for initiation, several types of explants: single node fragments without leaves, single node fragments with leaves, two-node fragments without leaves, two-node fragments with leaves, multiple node fragments (3-4 nodes with leaves) and 1-1.5 cm long shoot tips were tested on media of the same composition (modified Woody Plant Medium). After testing the explant types, three variants of nutritive media were tested: WPM with three concentrations of 2-Isopentenyladenine: 5, 10 and 20 mg/l in order to induce the proliferation of axillary buds in the initiation phase. In the multiplication phase, several types of culture vessels were tested, as well as the optimal number of inocula/vessel and the influence of growth regulators upon multiplication rate. Among the variants of nutritive media that were tested, modified WPM containing either 1 mg/l zeatin or 5 mg/l 2-Ip presented reasonable proliferation rates and relatively well-developed shoots. In the *in vitro* layering experiment, 2-Ip at 10 mg/l generated tens of thin shoots/explant. As gelling agents, starch and Plant Agar were tested. *Ex vitro* acclimation was carried out in Jiffy7 pellets in either covered or uncovered plastic trays and various survival rates (17-92 %) were obtained depending on explant types and culture conditions.

Keywords: cranberry, initiation, multiplication, acclimation, 2-Isopentenyladenine

INTRODUCTION

The North-American cranberry (*Vaccinium macrocarpon*) is a fruit shrub that has a great nutritional and medicinal importance due to its pleasant taste and its high vitamin and anthocyanin content. It was discovered that cranberry fruits play a role in cancer prevention and the destruction of cancer cells and that they have anti-inflammatory effect (Seeram, 2008; White *et al*., 2010). The bactericidal effect of the *Vaccinium macrocarpon* concentrate was tested on the bacteria present in ground meat, a great proportion of the bacteria in this product were destroyed (Wu *et al*., 2009). Cranberry juice also had a significant effect in preventing urinary infections with E. coli. (Perez-Lopez *et al.*, 2009).

The propagation of this species is done vegetatively; propagation by cuttings is cheap and efficient, as the mother plants generate a large number of runners that can be segmented into cuttings (Tomesh) that get rooted efficiently in a short time. Orthotropic shoots can also be an abundant source of cuttings, as the authors also experienced. Micropropagation has great practical importance for this species also, as this technology could be used for the rapid propagation of new, valuable genotypes, as well as for the conservation of some genotypes in artificially controlled conditions and to obtain a large amount of disease-free planting material in a short time, in controlled conditions, regardless of the season.

A small number of researchers investigated cranberry micropropagation (Marcotrigiano and McGlew, 1991; Debnath and McRae, 2001; Debnath, 2008) and of other *Vaccinium* species, such as the blueberry (Reed, 1991; Clapa *et al*., 2008; Fira *et al.*, 2008; Litwinczuk and Wadas-Boron, 2009), *Vaccinium vitis-idea* (Debnath, 2006; Ostrolucka *et al.*, 2004; Gajdosova *et al.*, 2006) and *Vaccinium angustifolium*, where effective *in vitro* propagation in RITA bioreactors was achieved (Debnath, 2009, 2011).

For the acclimation phase or the phase of direct *ex vitro* rooting together with acclimation, at the Fruit Research Station Cluj a new method was set up, based on floatation hydroculture, successfully used for blackberry *ex vitro* rooting and acclimation (Fira *et al*., 2011), also based on the method of *ex vitro* acclimation in liquid substrate (Fira and Clapa, 2009), also being inspired from the technology of "float hydroponics" (Ross and Teffeau, 1995; Tyson *et al*., 1999). This method was tested, in the present study, for the *ex vitro* acclimation of *Vaccinium macrocarpon.*

MATERIALS AND METHODS

Experiments for the initiation of cranberry (*Vaccinium macrocarpon*) *in vitro* cultures cultivar 'Pilgrim' were carried out, followed by experiments of *in vitro* multiplication and *ex vitro* acclimation.

1. The initiation of *in vitro* cultures was done starting from plants cultured in pots in the greenhouse. In all the initiation experiments the plant material was cut into 5-7 cm long fragments, washed under running water, rinsed with sterile deionized water (3 rinses) for 10 minutes per rinse phase. Under the laminar airflow hood, the plant material was disinfected with a mixture of 20 % ACE bleach in sterile deionized water, followed by repeated rinses with sterile deionized water (4 rinses).

The plant material was cut into several types of microcuttings, which were inoculated into test tubes containing about 5 ml of nutritive medium/tube. The test tubes were sealed with Folpack cling film and incubated in the growth room at about 2400 lux and the temperature of 23±3°C. Modified Woody Plant Medium (Tab. 1) was used, with various concentrations of 2-Ip.

Counts were done in order to establish survival rates and proliferation rates, as well as necrosis, contamination rates in the experimental variants. The inocula with growth and proliferation represent the microcuttings that generated several shoots/explant, the ones with growth and no proliferation represent the microcuttings that generated a single shoot/explant, in the majority of cases from the last bud from the apical part of the explant. The inocula with no growth were the ones that survived but did not generate any shoots. The inocula with necrosis were the ones that presented evident signs of necrosis but not contamination, whereas the inocula with contamination were the ones that presented evident signs of fungal or bacterial contamination. The apparent multiplication rates were estimated approximately, considering the number of 1.5 cm long fragments, presenting buds and leaves that could be excised and used as microcuttings in the multiplication phase. The basal, de-topped parts of the shoots regenerated in the initiation phase were not considered.

In the variants with two-node microcuttings with leaves and the ones with multiple-node microcuttings, counts and measurements were performed after 2 months of incubation, whereas in the variants with single-node microcuttings with or without leaves and two-node microcuttings without leaves the counts and measurements were performed after 4 months in culture, because after the first two months growth was very slow and very few microcuttings regenerated shoots.

Tab. 1

The composition of the nutritive medium used for cranberry *(Vaccinium macrocarpon*), cultivar 'Pilgrim' micropropagation

Component	Concentration
WPM* salts without FeNaEDTA	Full strength
FeNaEDDHA (Sequestrene 138)	100 mg/l
Myo-inositol	100 mg/l
Vitamin B_1	2 mg/l
Vitamin B_6	1 mg/l
Nicotinic acid	1 mg/l
Sugar	30 g/l
Growth regulators	2 Ip 5, 10, 20 mg/l sau zeatină- 2 mg/l
Plant-Agar	5 g/l
pH adjusted to 5	

* Woody Plant Medium

Experiment 1. In the first experiment, various types of inocula were tested in order to establish the optimal type of inoculum for initiation. Modified Woody Plant Medium (Lloyd & McCown) was used, with FeNaEDDHA as iron source and 5 mg/l 2-Ip (Tab. 1). The following variants were used as inocula: single-node fragments without leaves, single-node fragments with leaves, two-node fragments without leaves, two-node fragments with leaves, multiple-node fragments (3-4 nodes, with leaves), shoot tips 1 cm in length (with leaves). The plant material consisted of semi-hardwood fragments taken from about 1/3 of the apical portion of some orthotropic shoots from mature plants grown in the greenhouse.

Experiment 2. After the results of the first experiment and considering the obvious efficiency of the inocula consisting of shoot fragments with several nodes and, at the same time, considering the abundance of plant material consisting of plagiotropic shoots (runners), an experiment was carried out using WPM with three different concentrations of 2-Isopentenyladenine: 5, 10 and 20 mg/l. The aim was to induce the proliferation of axillary buds in the initiation phase. For each variant 40 test tubes were used. The plant material consisted of plagiotropic shoots (runners). About 20 % of the apical part of the runners was eliminated and the semi-hardwood and hardwood part of the runners from 50 % of the shoot length was excised. The inocula consisted of 3-node shoot fragments, with leaves.

2. Multiplication. The shoots regenerated in the initial phase were used for preliminary tests regarding *in vitro* multiplication. 2 cm-long shoot fragments with several nodes were used as inocula. Agar and starch were tested as gelling agents. 720 ml jars with screw caps, as well as Magenta GA_7 vessels were tested as culture vessels. The screw caps had 4 mm diameter vents with antibacterial filters made of autoclavable plastic sponge.

Experiment 3. An experiment was carried out in order to establish the optimal number of inocula/vessel in the multiplication phase. 370 ml jars with vented screw caps were used. The jars contained about 70 ml modified WPM medium/vessel, with 5 mg/l 2-Ip (Tab. 1). The two experimental variants consisted of vessels with 7, respectively 10 inocula/vessel. Each experimental variant had 4 replicates. The inocula consisted of 2 cm long microcuttings that resulted from the shoots regenerated in Experiment 2. The microcuttings were planted upright into the media, buried 2/3-3/4 deep into the media in order to ensure good contact between the axillary buds and the media. The culture cycle was of 3 months. One-way ANOVA was used for statistical analysis.

***Experiment 4* (*in vitro* layering)**. The vessel type, plant material and culture medium were the same as for Experiment 3. The concentration of 2-Ip was 10 mg/l. The explants were placed in plagiotropic position on the surface of the nutritive medium and then pushed gently in order to ensure good contact between the medium and the explant. One explant/vesel was used. The culture cycle was of 3 months.

Experiment 5. The effect of various concentrations of cytokinins upon proliferation rates and multiplication rates was tested. These were: Variant1- 1 mg/l zeatin + 6 g/l agar; Variant 2- 7 mg/l 2 Ip; Variant 3- 5 mg/l 2 Ip. The explants were 2 cm long shoot fragments and the vessels were 720 ml jars with vented screw caps, containing 100 ml/jar. Each experimental variant comprised 8 vessels with 15 inocula/vesel. The shoots regenerated from each plantlet were counted and the length of the shoots was measured. Proliferation rate was considered to be the number of shoots at least 1.5 cm in length and multiplication rate was established by calculating the number of 2 cm long microcuttings that could be excised/plantlet. The culture period was 3 months and a half.

3. Acclimation.

Experiment 6. *Ex vitro* acclimation was carried out in Jiffy7 pellets using various types of plant material: 3-4 cm long shoots resulting from Experiment 4; shoots regenerated in the initiation experiments on media with 5 mg/l 2-Ip, as well as shoots resulted from plants in the multiplication phase, cultured for 3 months on WPM with 5 mg/l 2-Ip. The Jiffy7 pellets were incubated in the growth room, in transparent Multi Purpose Trays covered with transparent lids in order to maintain air humidity. The culture period was of one month. The method of *ex vitro* acclimation in float hydroculture was also tested using plants resulted from multiplication on WPM with 5 mg/l 2-Ip. The latter were cut at a distance of 3-4 cm from their rooted base, thus a bunch of shoots was cut away from the base that contained the roots. The bunches of shoots as well as the rooted bases were transferred, separately, into floating cell trays in water with pH adjusted to 4.5 and cultured for 2 months.

RESULTS AND DISCUSSIONS

***In vitro* culture initiations.** The preliminary studies regarding the initiation of *in vitro* cultures of *Vaccinium macrocarpon* showed that the explants consisting of excised axillary buds presented the highest rates of necrosis. Single-node explants also gave very poor results regarding regeneration and growth. Multiple-node explants about 1 cm in length gave the best results regarding shoot regeneration of axillary shoots.

Experiment 1. The variants with inocula with multiple nodes, two-node explants with leaves and shoot tips grew very well in two months *in vitro*, generating vigorous shoots, with many leaves, some of these growing up to the test tube caps. The other variants were examined after 4 months in culture, because in the first two months they grew very little or not at all.

In the variant with multiple-node inocula, from the total of 100 microcuttings 66 presented growth with no proliferation (a single shoot grown from the apical part), 20 proliferated generating 2-4 shoots/explant and 14 microcuttings were apparently alive but did not regenerate shoots. Shoot lengths varied very much, some of them exceeding 10 cm (Fig.1).

The two-node inocula with leaves reacted in the following way: from the total of 58 inocula 32 grew but did not proliferate, 10 proliferated (2-4 shoots/explant) and 16 were apparently alive but without regeneration. The mode of growth and proliferation in this case was similar to that of the multiple node explants. Maximum shoot length was 9.5 cm.

In case of using 1 cm shoot tips for initiation, from the total of 30 inocula 16 grew without proliferation, 3 presented weak proliferation (2 shoots each), 8 did not grow and 3 died.

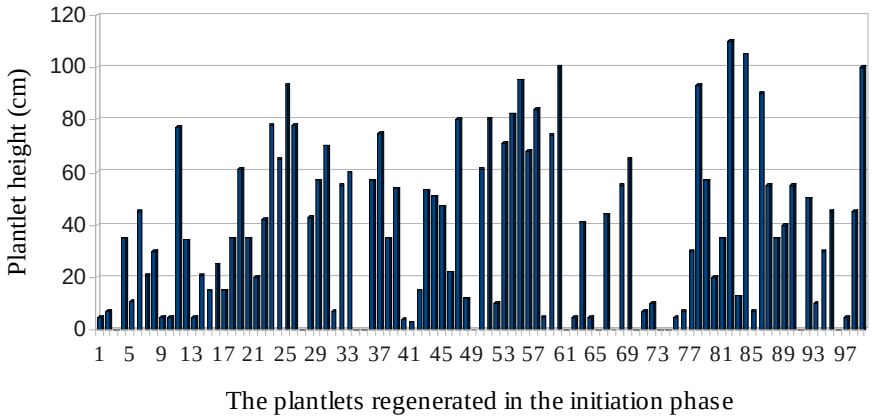

Fig. 1. Shoot lengths in the cranberry plantlets in the initiation phase

The variants examined after 4 months had a similar reaction: from the total of 50 single-node microcuttings without leaves, 26 regenerated shoots, 23 died and 1 got contaminated. From the 50 single-node microcuttings with leaves 25 regenerated and 25 died and from the 50 two-node microcuttings without leaves 33 died. Fig. 2 presents cultures of various types of microcuttings used for initiation.

Experiment 2. Increasing the concentrations of 2-Ip to 20 mg/l had, contrary to the expectations, inhibitory effect regarding shoot proliferation in the initiation phase. The reaction of the inocula was similar in the three experimental variants. Some regenerated several shoots, some regenerated only one shoot and the proliferation rate was low in all three cases (Fig. 3, Fig. 4.). There was a higher contamination rate than in the first experiment.

a b c

Fig. 2. Plantlets resulted from several types of explants used for initiation:
a. single-node with leaves; b. single-node without leaves; c. multiple node

2. Multiplication. After the preliminary results of the multiplication experiments we opted for agar as gelling agent and jars with vented metal caps as culture vessels.

Experiment 3. The use of 10 inocula/370 ml jar was found to be a viable option, as space was economized and a higher number of shoots/culture vessel resulted (Tab.2). The results regarding the total number of shoots/vessel were significant statistically ($p=0.0226$). The differences regarding proliferation rates were not significant statistically.

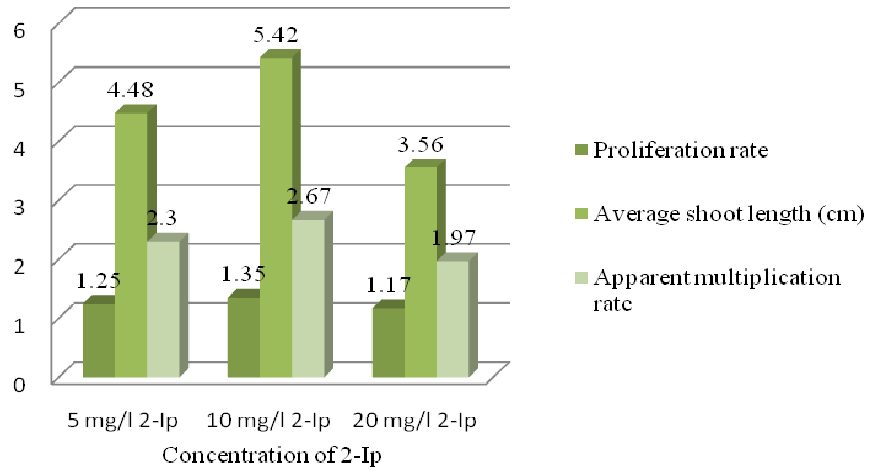

Fig. 3. The reaction of the inocula in the initiation phase in media with the 3 concentrations of 2iP

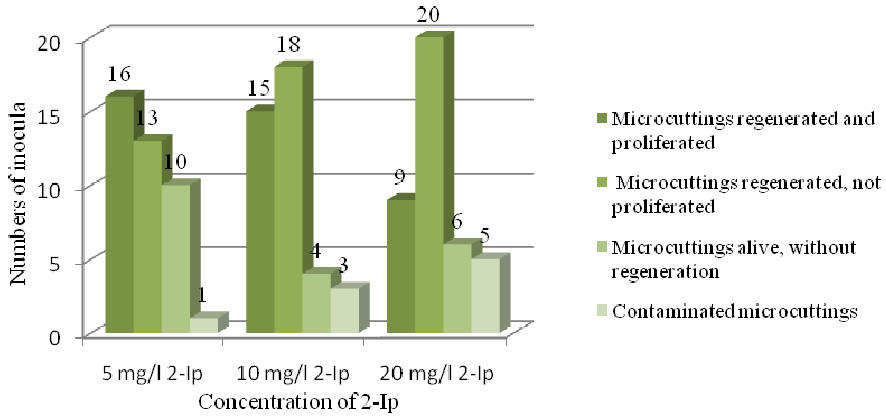

Fig. 4. The proliferation and multiplication rates on the three variants of initiation media

Experiment 4. In the experiment of *in vitro* layering the initial explant hypertrophied and got deformed on the surface of the medium, generating callus and a mass of buds and short, thin shoots that grew progressively in height, more and more buds appearing during the culture period (Fig.5). From the three culture vessels an average number of 34.33 shoots resulted, from which 19.33 were considered to be short (approximately 2 cm in length) and 15 were more than 2 cm in length.

Tab. 2

The average numbers of shoots obtained/vessel and proliferation rates

Variant	Average no. of shoots/vessel	Average proliferation rate
7 microcuttings/vesel	39.75	5.68
10 microcuttings/vesel	54.5	5.45

Experiment 5. From the total of 360 plantlets from 24 vessels 1781 shoots resulted. WPM with 5 mg/l 2-Ip gave superior results regarding all the characteristics studied, as compared with the other two variants (Fig.6). Table 3 presents the average values in all the characteristics studied. Morphologically, there were great similarities between the plantlets in the various experimental variants, nevertheless, the variant with 5 mg/l 2-Ip and the one with

1 mg/l zeatin yielded plantlets that looked healthier. In the variant with 7 mg/l 2-Ip there was larger basal callus and the shoots were more slender.

Fig. 5. Plantlets resulted from *in vitro* layering

Fig. 6. Plantlets in the multiplication phase

Tab. 3

Proliferation rates and multiplication rates in *Vaccinium macrocarpon* cultivar 'Pilgrim'

Variant	Average proliferation rate/variant	The sum of shoot lengths (cm), on the average, per vessel	Average shoot no./vessel/ variant	Average no. of inocula/vessel/ variant	Average multiplication rate/variant
1 mg/l zeatin	5.02	380.93	75.37	190.46	12.69
7 mg/l 2-Ip	4.75	456.25	71.37	228.13	15.2
5 mg/l 2- Ip	5.05	504.25	75.87	252.13	16.8

3. *Ex vitro* acclimation

Experiment 6. From the 40 shoots resulting from Experiment 4, 29 survived (72.5 %). The ones resulting from WPM with 5 mg/l 2-Ip the survival rates were of 80-90 %. In the shoots transferred *ex vitro* in float hydroculture the rooting and survival rate was 37.9 %, from the total of 240 shoots 91 got rooted, 8 were alive and not rooted and 141 were dead. From the 50 basal parts transferred *ex vitro* 47 survived (94 %). Fig. 7 presents shoots rooted in jiffy7 pellets and potted cranberry plants resulted from micropropagation.

Morphological peculiarities of the *in vitro* cultures of *Vaccinium macrocarpon*. In all the experiments of initiation and *in vitro* multiplication, in the beginning, few shoots grew from the inocula, in the majority of cases only one shoot/explant. The initial inoculum

hypertrophied and generated callus and buds as well as short shoots proliferated, which elongated progressively, one by one, during the 3-4 months of *in vitro* culture, the majority touching the lids of the culture vessels. Ed callus and hypertrophied and roots grew from the base of the plantlets and from some axillary buds in the regenerated shoots, frequently from buds ranging the basal part of the shoots until the middle of the shoots and sometimes from buds situated in the upper parts of the shoots. These roots grew in length, many of them touching the medium and in this contact zone the roots generated small, green, button-shaped calli, which later grew in size. These calli, after being transferred to fresh WPM with 5 mg/l did not regenerate any shoots or roots and died in a few weeks.

Fig. 7.a) Cranberry shoots rooted in jiffy7 pellets; b) Cranberry plants potted into peat

CONCLUSION

Vaccinium macrocarpon cultivar 'Pilgrim' can be successfully cultured *in vitro* on Woody Plant Medium (after Lloyd and McCown), the adequate growth regulators being 2-Isopentenyladenine and zeatin.

For *in vitro* culture initiation, as explants, multiple-node shoot fragments with intact leaves are recommended, as contamination rates are low and the axillary shoots grow to large sizes in a short time and practical results are obtained in 2 months.

The regeneration and growth of axillary shoots in the multiplication phase is slow, progressive, the axillary shoots are generated one after the other and they subsequently elongate fast, most of them growing up to the lid of the culture vessels and reasonable proliferating rates are obtained in 3-4 months of *in vitro* culture.

The glass jars with vented screw caps equipped with antibacterial filters proved to be more effective than Magenta GA_7 vessels.

Among the variants of nutritive media tested, modified WPM with 5 mg/l 2-Ip or 1 or 2 mg/l zeatin ensured reasonable proliferation rates and relatively well-developed shoots. In the *in vitro* layering, 10 mg/l 2-Ip generates tens of thin shoots/explant.

In the initiation as well as the multiplication phase adventitious roots are regenerated on the shoots. The roots that touch the nutritive medium generate calli that are apparently non-morphogenic.

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REFERENCES

1. Clapa D., A. Fira and T. Rusu (2008). The use of Isubgol and Sequestrene 138 for the *in vitro* propagation of the highbush blueberry (*Vaccinium corymbosum* L.). Journal of Food, Agriculture & Environment – JFAE Vol 6. (1), 132-134.
2. Debnath, S.C. (2006). Influence of propagation method and indole-3-butyric acid on growth and development of *in vitro*- and *ex vitro*-derived lingonberry plants. Canadian Journal of Plant Science, 235-243.
3. Debnath, S.C. (2011). Adventitious shoot regeneration in a bioreactor system and EST-PCR based clonal fidelity in lowbush blueberry (*Vaccinium angustifolium* Ait.). Scientia Horticulturae 128: 124–130.
4. Debnath, S.C. (2009 a). A two-step procedure for adventitious shoot regeneration on excised leaves of lowbush blueberry. *In Vitro* Cell. Dev. Biol-Plant 45:122-128.
5. Debnath, S.C. (2008). Zeatin-induced one-step *in vitro* cloning affects the vegetative growth of cranberry (*Vacinium macrocarpon* Ait.) micropropagules over stem cuttings. Plant Cell Tiss. Organ Cult. 93: 231-240.
6. Debnath, S.C. (2009 b). A Scale-up system for Lowbush Blueberry Micropropagation Using a Bioreactor. HortScience 44(7): 1962-1966.
7. Debnath, S. C. and K. B. McRae (2001). An Efficient *In Vitro* Shoot Propagation of Cranberry (*Vaccinium macrocarpon* Ait.) by axillary Bud Proliferation. *In Vitro* Cell Dev. Biol–Plant 37:243-249.
8. Fira, A., D. Clapa and C. Badescu (2008). Aspects Regarding the *In Vitro Propagation* of Highbush Blueberry Cultivar Blue Crop. Buletinul USAMV-CN, 65(1), 104-109.
9. Fira, A. and D. Clapa (2009). *Ex-Vitro* Acclimation of some Horticultural Species in Hydroculture. Bulletin UASVM, nr. 66(1), Horticulture, 44-51.
10. Fira, A., D. Clapa and E. Rakosy-Tican (2011). *In vitro* Propagation of the Thornless Blackberry Cultivar 'Loch Ness'. Bulletin UASVM Horticulture, 68(1).
11. Gajdosova, A., M.G. Ostrolucka, G. Libiakova, E. Odruskova and D. Simala (2006). Microclonal Propagation of *Vaccinium sp*. and *Rubus sp*. and detection of genetic variability of culture *in vitro*. Journal of Fruit and Ornamental Plant Research, 14(1):103-119.
12. Litwinczuk, W. and M. Wadas-Boron (2009). Development of Highbush Blueberry (*Vaccinium corymbosum* hort. Non l) *in Vitro* Shoot Cultures Under the Influence of Melatonin. Acta Sci. Pol. Hortorum cultus 8(3): 3-12.
13. Marcotrigiano, M. and S.P. McGlew (1991). A Two-stage Micropropagation System for Cranberries. J. Amer. Soc. Hort. Sci. 116(5):911-916.
14. Ostrolucka, M.G., G. Libiakova, E. Ondruskova and A. Gajdosova (2004). *In vitro* propagation of *Vaccinium* species. Acta Universitatis Latviensis, Biology, Vol. 676, 207-212.
15. Pérez-López, F.R., J. Haya and P. Chedraui (2009). *Vaccinium macrocarpon*: An interesting option for women with recurrent urinary tract infections and other health benefits. J. Obstet. Gynaecol. Res. Vol. 35, No. 4: 630–639.
16. Reed, B. M. (1991). The Use of Zeatin to Initiate *in Vitro* Cultures of *Vaccinium* Species and Cultivars. Hort Science 26 (10): 1320-1322.
17. Seeram, N.P. (2008). Berry Fruits for Cancer Prevention: Current Status and Future Prospects. J. Agric. Food Chem. 56: 630-635.
18. Tomesh, R. *Cranberry production*. University of Wisconsin, USA. USAID Agrobiznis Project (http://www.agrobiznis.net/documents/RS_Serbia%20Cranberry %20Production% 20PP.pdf).
19. White, B.L., L.R. Howard and R.R. Prior (2010). Polyphenolic Composition and Antioxidant Capacity of Extruded Cranberry Pomace. J. Agric. Food Chem. 58, 4037–4042.
20. Wu, V.C.H., X. Qiu, B.G. de los Reyes, C.S. Lin, and F.Y. Pan (2009). Application of cranberry concentrate (*Vaccinium macrocarpon*) to control *Escherichia coli* O157:H7 in ground beef and its antimicrobial mechanism related to the downregulated slp, hdeA and cfa . Food Microbiology 26: 32–38.