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THE GENETIC VARIABILITY EVALUATED WITH MOLECULAR MARKERS ON THE BILBERRY TISSUE LINES

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Abstract

The tissue lines derived from the Arieşeni, Retezat and Valea Sebeşului bilberry genotypes grown on a WPM medium supplemented with 40, 60 and 80 mg/l AS, were subject to molecular analysis through amplification of DNA samples with ISSR and RAPD primers. The results of agarose gel electrophoresis of the amplification products show the existence of significant differences in the model of polymorphic bands obtained from tissue lines, compared to the mother plant.
Based on the obtained results, the presence of somatic variability, induced in the tissue lines under the influence of AS, is emphasized. It consists in the absence of binding sites of the HB-12 ISSR marker compared to the mother plant. Considering the statements in the specialty literature, concerning the mutagenic effect of growth, we can state that AS caused changes within the DNA level in the bilberry calluses selected by us under the experimental conditions.

Keywords*: molecular analysis, bilberry tissue lines, variability.*

INTRODUCTION

Vaccinium myrtillus L. species provide raw materials to obtain a wide range of natural medicines (phytotherapeutic products), dietary supplements, natural dyes and preservatives. Worldwide there is a strong preference for the use of herbals detrimental drugs produced by chemical synthesis.
Techniques for in vitro cultivation of plant tissues and cells allow the obtaining and selection of proliferative tissue lines producing secondary metabolites. In order to establish a selection technology of cell lines producing biologically active substances (anthocyanins) by *in vitro* culture, cells response is tested on different tissue culture media with proper hormonal balance. The results demonstrate the appropriateness of such researches and open the possibility of developing a system for production of these secondary metabolites in greater quantities (Botau, 2009; Botău, 2009).
Analysis of variability induced by artificial culture of cells and tissues is quick and easy using DNA amplification techniques with different molecular markers. Koonjul et al. (1999), Iandolino et al. (2004), cited by Pop R. (2008) show that amplification of DNA fragments, especially RAPD method, is better by the inclusion of PVP in the mixture reaction of PCR. It will be appreciated that the polyphenols remaining in DNA template solution can inhibit DNA amplification, but by adding PVP, the polyphenols are absorbed.
In our experiments, we used two techniques based on PCR: RAPD and ISSR technics, in order to obtain a model of polymorphic DNA bands specific for the study of genetic variability in spontaneous blueberries tissue lines.

MATERIALS AND METHODS

Biologic material is represented by blueberry callus originated genotypes (local populations) Arieşeni, Valea Sebeşului and Retezat. Tissue lines (callus) with leaf and stem origin were subcultured on woody plant medium (WPM) supplemented with 40, 60 şi 80 mg/l AS and their growth was assessed by statistical calculation of variance (Ciulca, 2006).
Tissue lines were analyzed using molecular technics: DNA samples were amplified with ISSR primer HB 12 and RAPD one OPA 05,

chosen based on literature. Depending on the presence (1) or absence (0) of the polymorphic bands at blueberry tissue lines we appreciated their genetic variability compared with mother plant.

DNA isolation and purification was done using the modified CTAB method and the samples were amplified with the specific primers. For the amplification the Green Taq master mix (Promega) was used following the next programs: cycle profile for ISSR primer: 1 cycle at 94°C for 3 min followed by 45 cycles at 94 °C for 30 sec, 54°C for 45 sec and 72 °C for 2 min and for RAPD: 1 cycle at 95°C for 5 min followed by 45 cycles at 95 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min.

The mention DNA extraction method was selected, even if it is a very complex one, because it was very important to remove the polysaccharides, the polyphenols and other components which interfered with the amplification reactions. Besides, 1% Polyvinylpyrrolidone (PVP) was added in the amplification mixtures to improve the reactions.

The samples amplification was done in three repetitions for each primer. The same primers were used for the genotyping of the three blueberries ecotypes, obtaining the same fingerprint each time.

RESULTS AND DISCUTIONS

1. **Results regarding the callus growth in subculture**

The results presented in table 1 show that the Valea Sebeşului population registered the highest values of callus growth in subculture on WPM medium supplemented with 60 mg/l AS, with a mean of 2.83 g, followed by population Arieşeni on the same hormonal balance, with values of 2.78 g for the stem callus.

The Retezat population shows a mean value of 2.70 g callus weight.

Considering the combined effect of the three factors (table 1) on blueberry callus grown in subculture, at Arieşeni population it was observed an amplitude of variation of 0.57 g with 2.21 g boundaries for leaf callus under the influence of hormonal balance 1.5 mg / l NAA 1.5 mg / l BAP + 40 mg / l AS and 2,78 g boundaries for stem callus subcultured on medium WPM supplemented with 1,5 mg/l ANA + 1,5 mg/l BAP + 60 mg/l AS.

Table 1. *The effect of the genotype, callus origin and AS concentration on the growth of bilberry callus subcultured on medium WPM (1,5 mg/l ANA+1,5 mg/l BAP)*

Genotype	**Arieşeni**	
Concentration (AS)	Callus origin	
	Leaf	**Stem**
40 AS	y2,21 b	x2,54 b
60 AS	y2,50 a	x2,78 a
80 AS	x2,26 b	x2,38 b
Genotype	**Retezat**	
Concentration (AS)	Callus origin	
	Leaf	**Stem**
40 AS	y1,92 b	x2,35 b
60 AS	y2,33 a	x2,70 a
80 AS	y2,20 ab	x2,51 ab
Genotype	**Valea Sebeşului**	
Concentration (AS)	Callus origin	
	Leaf	**Stem**
40 AS	y2,35 a	x2,62 a
60 AS	y2,56 a	x2,83 a
80 AS	x2,34 a	x2,34 b

$DL_{5\%}$=0,23g $DL_{1\%}$=0,31g $DL_{0,1\%}$=0,40g

The concentration of 60 mg/l AS positive significantly influenced the blueberry callus growth in subculture, regardless of its origin, with significant differences compared to the other AS concentrations tested. Under influence of this concentration (60 mg/l AS) were registered too significantly differences between calli with different origins, stem callus producing the best values.

Callus growth at Retezat population was strongly influenced by AS concentration and callus origin, there is significant differences between leaf and stem calli. Stem callus grew better under concentration 60 mg/lAS influence.

Valea Sebeşului blueberry population registered the best growth values in stem callus subculture under concentration of 60 mg/l AS influence, recording significant differences between 80 mg/l AS (where lower values were obtained) and the others two concentrations (40, 60 mg/l AS). The highest values were observed at variants with content of 60 mg/l AS by stem callus cultivation at populations: Valea Sebeşului (2,8 g), Arieşeni (2,7 g) and Retezat (2,6 g) (Figure. 1).

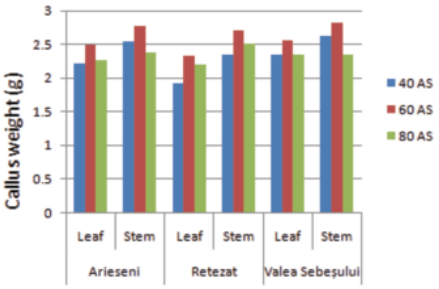

Figure 1. *The callus growth in subculture (30 days), on solid WPM medium.*

2. Results regarding the genetic variability on the tissue lines level evaluated with molecular markers

The tissue lines derived from the Arieşeni, Retezat and Valea Sebeşului genotypes grown on a media supplemented with 40, 60 and 80 mg/l AS, were subject to molecular analysis through amplification of DNA samples with the ISSR HB 12 primer.

The results of agarose gel electrophoresis of the amplification products showed the existence of significant differences in the model of polymorphic bands obtained from tissue lines, compared to the mother plant (Figure 2).

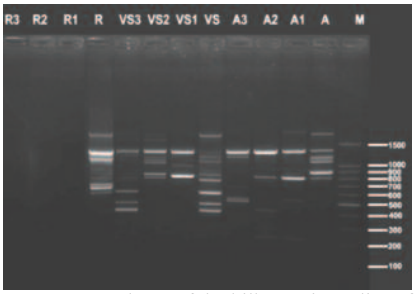

Figure 2. PCR analyses of the bilberry tissue lines from Arieşeni, Valea Sebeşului and Retezat populations with the ISSR marker HB12

M – molecular marker (100-1500 pb), A- mother plant Arieseni, A1 tissue line 40 mg/l AS, A2 tissue line 60 mg/l AS, A3 tissue line 80 mg/l AS, VS Mother plant Valea Sebesului, VS1 tissue line 40 mg/l AS, VS2 tissue line 60 mg/l AS, VS3 tissue line 80 mg/l AS, R- Mother plant Retezat, R1 tissue line 40 mg/l AS, R 2 tissue line 60 mg/l AS, R 3 tissue line 80 mg/l AS

A lack of bands can be observed, corresponding to DNA fragments with the length between 800 -1300bp and those with the length of 600-100 bp, at tissue lines grown in concentrations of 40 and 60 mg/l AS, from both the Arieseni population and the Valea Sebeşului population. The tissue lines grown in the presence of 80 mg/l AS have a pattern of bands different from the other lines and from the mother plants, at the Valea Sebeşului and Arieseni genotypes. The tissue lines derived from the Retezat genotype showed no pattern of polymorphic bands. The lack of bands suggests that the DNA lost the binding sites for the ISSR HB-12 marker.

Based on the obtained results, the presence of somatic variability, induced in the tissue lines under the influence of AS, is emphasized. It consists in the absence of binding sites of the HB-12 ISSR marker compared to the mother plant. Considering the statements in the literature, concerning the mutagenic effect of growth regulators (Marele, 2009; Vicas, 2009) we can state that AS caused changes within the DNA level in the bilberry calluses selected by us under the experimental conditions.

A total number of 6 tissue lines derived from the Retezat genotype, grown under the presence of three concentrations of AS (40, 60, 80 mg/l), were subject to molecular analysis using RAPD OPA 05 marker. The pattern of polymorphic bands (Figure 3.) indicates the existence of somatic variability at the level of tissue lines compared with the mother plant. It can be observed that at the same concentration of AS (40 mg/l), tissue lines are different: one reveals four bands and the other has none. At the concentration of 60 mg/l AS no polymorphic bands have been registered.

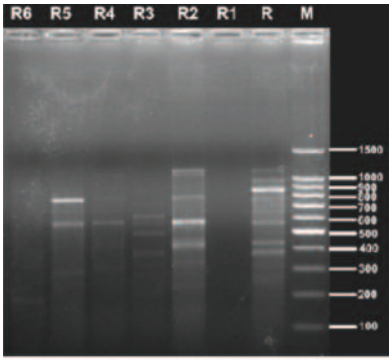

Figure 3. PCR analyses of the bilberry tissue lines from Retezat population with RAPD OPA 05 marker

M - Molecular marker (100-1500 pb), R- Mother plant Retezat, R1 tissue line 40 mg/l AS, R 2 tissue line 60 mg/l AS, R 3 tissue line 80 mg/l AS.

The tissue lines grown under the presence of 80 mg/l AS are also different: one has two bands

and the other has none. Unlike the mother plant, the lack of bands in some tissue lines indicates the lack of DNA binding sites for the marker OPA 05. These results demonstrate that AS cause changes at the DNA level in the selected tissue lines, cultivated *in vitro*.

CONCLUSIONS

To obtain an abundant growing callus tissue, at spontaneous bilberry, *we recommend* the utilization of Retezat, Arieşeni and Valea Sebeşului genotypes, from stem explants grown on solid WPM media, supplemented with a uniform ratio between auxinic ANA and BAP citokinine and a concentration of 60 mg /l AS.

The origin of callus influences the growth ability of callus in subculture. Stem callus grows better than the leaf callus, and there are significant differences between them.

The obtained results revealed a relatively high genetic polymorphism at the level of the studied spontaneous bilberry genotypes and good discriminatory power of the RAPD and ISSR techniques. These analyses can be used to determine genetic differences at spontaneous bilberry, allowing the determination of genetic differences between populations.

For carrying out the molecular analyses *we recommend* the utilization of the primers **HB-12, HB-15** and **UBC 818**, which revealed the highest rates of polymorphism (100%).

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