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ORGANOGENESIS *IN VITRO* FROM THE LEAF OF BLACKBERRY CV ČAČANSKA BESTRNA

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The paper presents evaluation of different types and concentrations of plant growth regulators on induction of adventitious organogenesis from the leaf of blackberry cultivar Čačanska Bestrna. This genotype was introduced and maintained in tissue culture on Murashige and Skoog medium (MS) (1962), mineral salts and organic complex with in mg l^{-1}: BAP 1.0, IBA 0.1, GA_3 0.1. Young, expanded leaves taken from upper third of in vitro proliferating shoots were used as initial explants. The leaves were cut three times across the midrib and placed with adaxial side touching the regeneration medium. In this experiment 20 types of regeneration media have been evaluated with different types and concentrations of cytokinins and auxins: BAP (2.0 mg l^{-1}) or TDZ (1.0 mg l^{-1}) alone, or each in combination with different concentrations of IBA, NAA and 2,4-D (0.1, 1.0 and 2.0 mg l^{-1}).

In cv Čačanska Bestrna regeneration of adventitious shoots was observed on three different types of media with BAP combined with IBA (0.1 and 1.0 mg l^{-1}) and NAA (0.1 mg l^{-1}). TDZ alone or in combination with IBA (0.1, 1.0 and 2.0 mg l^{-1}) and NAA (0.1 mg l^{-1}) has been more

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effective at induction of regeneration than BAP. The highest percentage of regeneration was obtained precisely on medium supplemented with TDZ alone (41.66%).

Key words: Cv Čačanska Bestrna, adventitious organogenesis, leaf explants, cytokinins, auxins

INTRODUCTION

Some of plant cell and tissue culture techniques, applied either individually or combined with the recombinant DNA technology, are more widely used today, as they have proved to be powerful in crop improvement. Their application is based on possibility of regeneration of plants from cultured cells, tissues and/or organs. It is possible to provide development of shoots, roots or somatic embryos either directly from differentiated cells or indirectly from callus or cell suspension cultures (HICKS, 1980).

By modification of conditions of *in vitro* tissue culture it is possible to trigger new morphogenetic processes, whereas application of exogenous plant growth regulators plays a crucial role in the induction of specific developmental patterns (THORPE, 1994).

In vitro adventitious organogenesis has been studied in different genotypes of genus *Rubus*, including blackberry, raspberry and their hybrids (COUSINEAU and DONELLY, 1991; SWARTZ *et al*., 1990., MCNICOL and GRAHAM, 1990., FIOLA *et al*., 1990., GRAHAM *et al*., 1997; MEZZETTI *et al*., 1997; MENG *et al*., 2004; TURK *et al*., 1994; ZAWADZKA and ORLIKOWSKA, 2006). Nevertheless, no efficient regeneration protocol that would have wider application within this genus has been developed so far. Performed investigations have shown that the efficacy of regeneration depends greatly on numerous factors such as: hormonal (type and concentration of plant growth regulators) and mineral composition of medium, age and type of explants (leaves, petioles, internodes, cotyledons or mature embryos), origin of explants (*in vitro* or *in vivo*), explant orientation during regeneration, and as well as incubation conditions (length of incubation, photoperiod, light intensity, temperature). However, in all cases, efficacy of regeneration varied widely among genotypes which implies necessity to optimize conditions of adventitious organogenesis for each individual genotype. Thus, study of the impact of hormonal composition of a medium on adventitious organogenesis in eight genotypes (3 blackberry cultivars and 5 red raspberry cultivars) within *Rubus* genus (GRAHAM *et al*., 1997) have shown that blackberry has larger regenerative potential in comparison with raspberry. Nevertheless, although the efficacy of regeneration within one specific genotype varied with different hormonal composition of a medium, the usage of RAPDs markers suggested that the smaller genetic distance between evaluated genotypes the more similar their regenerative potential on media with identical hormonal composition.

Bearing in mind the fact that the efficacy of regeneration in *in vitro* culture is primarily determined by relation genotype – hormonal composition of a

medium, the objective of the study was investigation of influence of different types and concentrations of plant growth regulators on induction of adventitious organogenesis from leaf in blackberry cultivar Čačanska Bestrna.

MATERIAL AND METHODS

Cv Čačanska Bestrna (*Rubus fruticosus* L.) was used as a model plant. It resulted from the breeding work which included application of planned hybridization, the process initiated at the Fruit Research Institute Čačak in the 80's of the XX century. It derived from the cross of cvs Dirksen Thornless x Black Satin in 1984. It was selected in 1987, and tested as hybrid designated as 1/III/87. The cultivar is thornfree, just like its parental combination. It has performed outstandingly in respect of cropping, fruit quality, resistance to diseases and early spring frosts (STANISAVLJEVIĆ, 1999). It also shows high potential for vegetative propagation, even by micropropagation (RUŽIĆ and CEROVIĆ, 1998; RUŽIĆ and LAZIĆ, 2006).

This genotype has been introduced in tissue culture and maintained on MURASHIGE and SKOOG (MS) medium (1962), mineral salts and organic complex, with in mg l^{-1}: 6-benzylaminopurine (BAP) 1,0, indole-3-butyric acid (IBA) 0,1 and giberelic acid (GA_3) 0,1.

Young, fully expanded leaves from upper thirds of in vitro propagated shoots were used as initial explants. The leaves were cut three times across the midrib, placed with adaxial side touching the regeneration medium poured into 9-cm petry dishes (50 ml of the medium). The experiment included twenty different regeneration media with different types and concentrations of cytokinins and auxins: BAP or N-pheny-N'-1,2,3-thidiazol-5-ylurea (thidiazuron, TDZ), either individually or combined with different concentrations of IBA, α-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) (Table 1). All media had mineral salts and organic complex according to MS with 20 g l^{-1} sucrose and 7 g l^{-1} of agar. pH value of a medium was adjusted to 5.7 with 0.1 N KOH prior to autoclaving.

The plants were kept in the growth chamber with controlled temperature (23±1^{o}C), photoperiod (16/8 h, light/dark), light intensity on plant surface being 8.83 Wm^{-2}, provided with cool white fluorescent tubes of 40W, 6.500^{o}K.

Percentage of regeneration and average number of regenerants per explant for each medium were determined upon 45-day maintenance in tissue culture. The data (average number of regenerants per explant) were analysed by Duncan's Multiple Range Test at $p<0.05$.

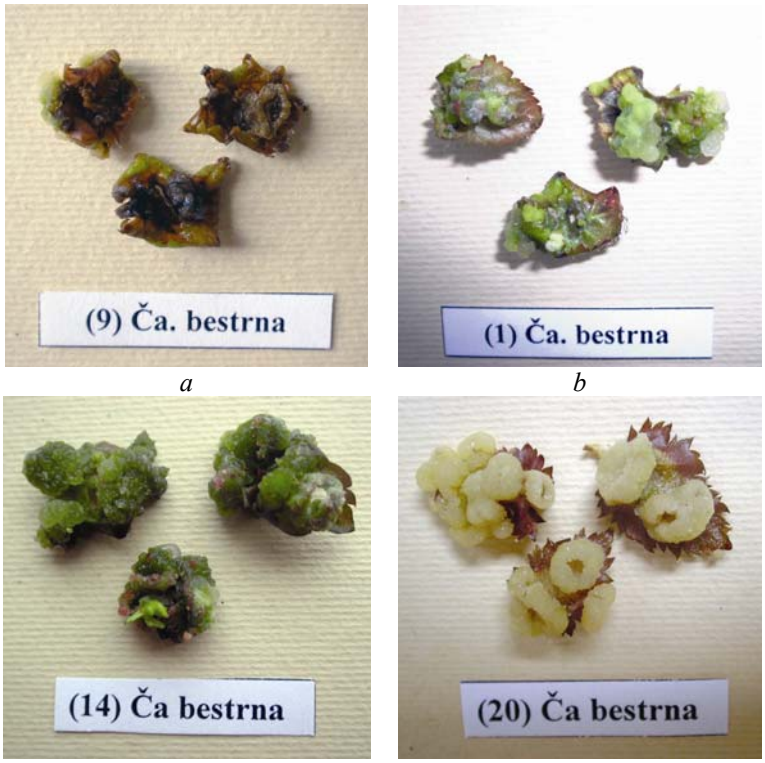
Table 1. - Hormonal composition of regeneration media

Medium designation	BAP (mg l^{-1})	TDZ (mg l^{-1})	IBA (mg l^{-1})	NAA (mg l^{-1})	2,4 D (mg l^{-1})	GA_3 (mg l^{-1})
1	2,0	-	-	-	-	
2	2,0	-	0,1	-	-	0,1
3	2,0	-	1,0	-	-	0,1
4	2,0	-	2,0	-	-	0,1
5	2,0	-	-	0,1	-	-
6	2,0	-	-	1,0	-	-
7	2,0	-	-	2,0	-	-
8	2,0	-	-	-	0,1	-
9	2,0	-	-	-	1,0	-
10	2,0	-	-	-	2,0	-
11	-	1,0	-	-	-	-
12	-	1,0	0,1	-	-	0,1
13	-	1,0	1,0	-	-	0,1
14	-	1,0	2,0	-	-	0,1
15	-	1,0	-	0,1	-	-
16	-	1,0	-	1,0	-	-
17	-	1,0	-	2,0	-	-
18	-	1,0	-	-	0,1	-
19	-	1,0	-	-	1,0	-
20	-	1,0	-	-	2,0	-

RESULTS AND DISCUSSION

After 45 days of culturing, the percentage of callus formation on leaves amounted to 100% for all evaluated media. On media supplemented with BAP alone or in combination with 2,4-D leaf necrosis and dieback of remaining leaves were observed (Fig. 1a), while on all other media, leaves maintained viable over the evaluated period. In addition, red leaf pigmentation, particularly pronounced at the leaf edge, was recorded as well. Appearance and quantity of the formed calli was also dependent on type and concentration of applied auxin. The lowest callus production was observed on media supplemented with either BAP or TDZ alone (Fig. 1b). Addition of IBA and NAA into regeneration media increases the amount of formed calli which had very similar consistency in both auxins. Namely, the calli were dark green, firm and were formed abundantly at the cross-sectional area of both midrib and petiole (Fig. 1c). Such influence of different types and concentrations of cytokinins on caulogenesis was also observed in other fruit varieties (RUŽIĆ *et al*., 1991; BOŠKOVIĆ and RUŽIĆ, 1996). Adding 2,4-D into regeneration medium brought about formation of loose callus mass of light green

colour which, with increase of concentration of this auxine, took on either dark brown on media with BAP, or light yellow on media with TDZ (Fig. 1d).



a *b*

c *d*

Fig. 1. – Calli of cv Čačanska Bestrna on media with different hormonal composition: a) 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} 2,4-D; b) 2.0 mg l^{-1} BAP; c) 1.0 m gl^{-1} TDZ + 2.0 mg l^{-1} IBA; d) 1.0 m g l^{-1} TDZ + 2.0 mg l^{-1} 2,4-D

BAP and TDZ (substituted urea with cytokinin like activity) are the most commonly used cytokinins for induction of regeneration of different *Rubus* genotypes (COUSINEAU and DONELLY, 1991; McNICOL and GRAHAM, 1990.; FIOLA *et al*., 1990., GRAHAM *et a*l., 1997; MEZZETTI *et al*., 1997; MENG *et al*., 2004). The results obtained imply that TDZ, applied either individually or combined with IBA (0.1, 1.0 and 2.0 mg l^{-1}) and NAA (0.1 mg l^{-1}), is more efficient in induction of regeneration than BAP (Table 2). Major perecentage of regeneration (41.66%) has been obtained precisely on medium supplemented with TDZ alone (Fig. 2). Also, thidiazuron was significantly more effective than BAP at inducing adventitious organogenesis in some representatives of genus *Malus* (KORBAN and O'CONNOR, 1992), *Pyrus* (CHERVEAU and SKIRVIN, 1992 cited according to TANG *et al.*, 2002), *Prunus* (ESCALETTES and DOSBA, 1993; ESPINOSA

et al, 2006) and *Rubus* (FIOLA *et al*., 1990; COUSINEAU and DONNELLY, 1991). Applied concentration of TDZ (1.0 mg l^{-1}) in this experiment resulted in pretty high percentage of regeneration in cv Čačanska Bestrna, as compared to the results of FIOLA *et al*. (1990) who, with similar concentration of TDZ (5μM) in blackberry cvs Loch Ness and Shawnee, obtained only 8% of regeneration. Increase in TDZ concentration up to the 50 μM did not significantly change the percentage of regeneration from leaves in the stated cultivars. In contrast to that, GRAHAM *et al*. (1997) claim that TDZ in cvs Hull Thornless, Loch Ness and Chester may induce adventitious organogenesis only at concentrations higher than 9 μM. It implies on genetic specifity toward growth regulators.

Table 2. – Efficacy of regeneration from leaf of cv Čačanska Bestrna on media with BAP and TDZ

Medium designation	Percentage of explants with adventitious shoots	Average number of regenerants per explant
1.	0	-
2.	20,83	2,2 bc
3.	4,16	1,0 d
4.	0	-
5.	12,5	2,7 abc
6.	0	-
7.	0	-
8.	0	-
9.	0	-
10.	0	-
11.	41,66	1,9 c
12.	37,50	2,8 ab
13.	37,50	2,1 bc
14.	33,33	3,2 a
15.	12,50	2,0 bc
16.	0	-
17.	0	-
18.	0	-
19.	0	-
20.	0	-

Means in the same column that are followed by different letters are significantly different ($p \leq 0.05$) using Duncan's Multiple Range Test.

Adding auxins into regeneration medium with TDZ either decreases percentage of regeneration, in dependence on concentration of the applied auxine (IBA or NAA), or inhibits it completely (2,4-D). Increase of IBA rate brings about reduction of explants that produce adventitious buds. Nevertheless, the average

number of regenerants per explant is increased, resulting in the highest average number of regenerants per explant (3.2) precisely on medium with in 1.0 mg l^{-1} TDZ and 2.0 mg l^{-1} IBA (Table 2, Fig. 3). On the other hand, application of NAA at concentrations 1.0 and 2.0 mg l^{-1} fully inhibits organogenesis. Research performed by GRAHAM et al. (1997) showed that in three blackberry cultivars NAA is more effective auxin than IBA, as regards induction of adventitious organogenesis. However, applied rates of NAA were substantially lower than those applied in this trial, and they ranged from 0.067-0.27 μM, whereas optimal rate of this auxin in all three genotypes amounted to 0.13 μM, regardless of TDZ rate.

Some studies on influence of cytokinins on adventitious organogenesis from leaf in different blackberry cultivars suggested that BAP largely influences induction of regeneration if applied individually at concentrations of 5-10 μM, or combined with 0.5 μM IBA (MEZZETTI et al., 1997; GRAHAM et al., 1997; MENG et al., 2004). Nevertheless, this trial has shown that BAP at concentration of 2.0 mg l^{-1} makes no influence on induction of adventitious organogenesis in cv Čačanska Bestrna if applied individually, without auxins (Table 2). The highest regeneration rate was obtained on the medium that, besides BAP, contained IBA at concentration of 0.1 mg l^{-1}, which is in accordance with the results of the aforementioned authors (Fig. 4). Increase of the rate of IBA to 1.0, i.e. 2.0 mg l^{-1} considerably reduces regeneration rate (4.16%) or, more importantly, inhibits it completely. Adding NAA as the auxins source (at concentration of 0.1 mg l^{-1}) into the regeneration medium with BAP results in considerably lower regeneration rate (12.5%). The result is in compliance with those obtained by MENG et al. (2004) who found that, in blackberry cv Marion, IBA was significantly more effective in inducing shoot regeneration as compared to IAA, NAA and 2,4-D.

Complete inhibitory effect of 2,4-D on adventitious organogenesis in cv Čačanska Bestrna suggests that this auxine is not aplicable in the regeneration trials under described experimental conditions.

Fig. 2, Fig. 3, Fig. 4

Fig. 2. – Regenerated shoots on medium with 1.0 mg l^{-1} TDZ

Fig. 3. - Regenerated shoots on medium with 1.0 mg l^{-1} TDZ and 2.0 mg l^{-1} IBA

Fig. 4. - Regenerated shoots on medium with 2.0 mg l^{-1} BAP and 0.1 mg l^{-1} IBA

CONCLUSION

The results obtained in this experiment infer the following:

- Efficacy of regeneration in cv Čačanska Bestrna is preconditioned both with type of cytokinins and cytokinin/auxine ratio;
- TDZ applied either alone or in combination with different types and concentrations of auxins has proved to be more efficient than BAP as regards induction of adventitious organogenesis *in vitro* in this genotype. The highest regeneration rate was obtained precisely on medium supplemented with TDZ alone (41.66%);
- BAP has no influence on induction of adventitious organogenesis in this genotype if applied individually, without auxins (IBA and NAA);
- Increase of rates of IBA and NAA in regeneration media reduces efficacy of regeneration both with TDZ and BAP;
- Adding 2,4-D into regeneration media fully inhibits adventitious shoot formation.

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ORGANOGENEZA *IN VITRO* IZ LISTA KUPINE CV ČAČANSKA BESTRNA

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Izvod

U radu je ispitan uticaj različitih vrsta i koncentracija biljnih regulatora rastenja na indukciju adventivne organogeneze iz lista kod kupine cv Čačanska bestrna. Ovaj genotip je uveden u kulturu i održavan na medijumu Murashige i Skoog (MS) (1962) mineralne soli i organski kompleks sa u mg l^{-1}: BAP 1,0, IBA 0,1, GA_3 0,1.

Kao početni eksplantati korišćeni su mladi, dobro razvijeni listovi sa gornje trećine izdanaka umnoženih in vitro. Listovi su tri puta poprečno parani u odnosu na glavni lisni nerv i postavljani adaksialnom stranom na medijum za regeneraciju. U eksperimentu je ispitano 20 medijuma za regeneraciju sa različitim vrstama i koncentracijama citokinina i auksina: BAP (2 mg l^{-1}) ili TDZ (1 mg l^{-1}), samostalno ili u kombinaciji sa različitim koncentracijama IBA, NAA i 2,4 D (0,1, 1,0 i 2,0 mg l^{-1}).

Regeneracija adventivnih izdanaka kod cv Čačanska bestrna je dobijena na 3 različita tipa medijuma sa BAP u kombinaciji sa IBA (0,1 i 1,0 mg l^{-1}) i NAA (0,1 mg l^{-1}). TDZ je samostalno, ili u kombinaciji sa IBA (0,1, 1,0 i 2,0 mg l^{-1}) i NAA (0,1 mg l^{-1}) pokazao mnogo veću efikasnost u indukciji regeneracije nego BAP. Najveći procenat regeneracije je upravo i dobijen na medijumu koji je sadržavao samo TDZ.

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