Shoot proliferation ability of selected cultivars of *Rubus* spp. as influenced by genotype and cytokinin concentration

Vplyv genotypu a koncentrácie cytokinínu na schopnosť proliferácie výhonkov pri vybraných kultivaroch *Rubus* spp.

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Abstract

The aim of this work was to develop protocols for micropropagation of selected *Rubus* cultivars – 'Tulameen' and 'Black Jewel' (*Rubus idaeus* L.), 'Black Satin' (*Rubus fruticosus* L.) and 'Tayberry' (*Rubus fruticosus* x *Rubus idaeus*). Nodal segments carrying dormant buds were used for *in vitro* culture establishment. For shoot initiation, MS medium containing 8 g*L^{-1} phytoagar, 30 g*L^{-1} sucrose, 1 mg*L^{-1} 6-benzylaminopurine (BAP), 0.2 mg*L^{-1} indole-3-butyric acid (IBA) and 150 mg*L^{-1} cefotaxime was used. The shoot proliferation ability of each cultivar was evaluated by multiplication coefficient (number of shoots formed per explant) during 4 subcultures. Statistical analyses confirmed the significantly higher shoot multiplication ability in cvs. 'Black Satin' and 'Tulameen' in comparison with cvs. 'Tayberry' and 'Black Jewel', as well as significant differences in shoot proliferation between subcultures (1st and 2nd versus 3rd and 4th). Testing of influence of different BAP concentrations on shoot formation showed that BAP 1-2 mg*L^{-1} is the best for shoot proliferation with the higher multiplication rate obtained in cv. 'Black Satin' in comparison with 'Black Jewel'. Statistical evaluation showed that genotype and BAP concentration significantly influenced the ability of shoot proliferation in *Rubus* cultivars.

Keywords: cytokinin, genotype, micropropagation, *Rubus*

Abstrakt

Cieľom výskumu bolo vyvinúť protokol pre mikropropagáciu vybraných kultivarov *Rubus* – 'Tulameen' a 'Black Jewel' (*Rubus idaeus* L.), 'Black Satin' (*Rubus fruticosus* L.) a 'Tayberry' (*Rubus fruticosus* x *Rubus idaeus*). Pre založenie *in vitro* kultúr boli použité nodálne segmenty nesúce dormantné púčiky. Iniciácia rastu výhonkov prebiehala na MS médiu s obsahom 8 g*L^{-1} fytoagaru, 30 g*L^{-1} sacharózy, 1 mg*L^{-1} 6-benzylaminopurín (BAP), 0.2 mg*L^{-1} kyselina indolyl-3-maslová (IBA) a 150 mg*L^{-1} cefotaxímu. Schopnosť proliferácie výhonkov jednotlivých kultivarov bola hodnotená pomocou koeficientu multiplikácie (počet výhonkov vytvorených na explantát) počas 4 subkultivácií. Štatistické analýzy potvrdili významne vyššiu schopnosť multiplikácie výhonkov pri kultivaroch 'Black Satin' a 'Tulameen' v porovnaní s 'Tayberry' a 'Black Jewel', ako aj významné rozdiely v proliferácii výhonkov medzi subkultiváciami (1. a 2. versus 3. a 4.). Testovanie vplyvu rôznych koncentrácií BAP na tvorbu výhonkov ukázalo, že BAP 1-2 mg*L^{-1} je najlepší pre proliferáciu výhonkov s vyššou hodnotou multiplikácie pri kultivare 'Black Satin' v porovnaní s 'Black Jewel'. Štatistické hodnotenie ukázalo, že genotyp a koncentrácia BAP štatisticky významne ovplyvnili schopnosť proliferácie výhonkov pri kultivaroch druhov *Rubus*.

Kľúčové slová: cytokinín, genotype, mikropropagácia, *Rubus*

Introduction

Rubus is a large genus of flowering plants of the family *Rosaceae*. It includes hundreds of species and numerous natural hybrids, as either hybrids produced by breeding. *Rubus* genus is widespread in Europe, North America and west Asia (Jennings et al., 1991). Raspberries and blackberries belong to the oldest and most widely grown fruit plants. Fruits are well-known through their health benefits, as they contain high levels of antioxidants, anthocyanins and other polyphenols. The demand for this fruit is continuously growing, especially in the food industry for products as juices, jams, syrups, etc. (Moyer et al., 2002).

In the past, raspberries, blackberries, as well as billberries and lingonberries were covering large geographical areas in mountain and sub-mountain regions of Slovakia. But increased demand on these small fruits led to the uncontrolled harvesting, what resulted in devastation of these natural areas. Berry production did sharply decrease during the 1990s, but the demand is continuously rising not only among home gardeners and small farmers, but also for commercial production. However, some berry cultivars recently introduced into Slovakia are potentially profitable alternative as non-conventional berry crops. Among them are cultivars of the raspberry (*Rubus idaeus* L.), blackberry (*Rubus fruticosus* L.) and their hybrids, high-bush blueberry (*Vaccinium corymbosum* L.) and lingonberry (*Vaccinium vitis-idaea* L.). Large-scale cultivation of selected cultivars of these species can greatly help solve the problem of devastation of natural population of wild species and contribute to the biodiversity restoration.

Raspberries and blackberries can be commercially propagated by the classical methods of vegetative propagation, i.e. by hardwood and soft wood cuttings, by layering and bush division. However, success of these methods is limited. They are time-consuming, can support spread of pathogens and do not provide efficient rooting of the plantlets (Najaf-Abadi and Hamidoghli, 2009).On the other hand, tissue-culture can provide significant amount of stock material for commercial propagation. Obtained plans are high quality and genetically stable (Gajdošová et al., 2006). However, it is important to say, that micropropagation requires a lot of experimental work for an optimization of all its phases (initiation, multiplication, rooting), not to mention difficult acclimatization to *ex vitro* conditions afterwards. A number of reports have been published for *Rubus* spp. micropropagation, either from shoot tips (Bobrowski et al., 1996), or nodal segments (Gonzales et al., 2000). Dormant axillary and apical buds are also often used as primary explants (Ružić and Lazić, 2006).

The aim of this study was to develop protocols for micropropagation of selected *Rubus* cultivars - 'Tulameen' and 'Black Jewel' (*Rubus idaeus* L.), 'Black Satin' (*Rubus fruticosus* L.) and 'Tayberry' (*Rubus fruticosus* x *Rubus idaeus*). The comparison of shoot multiplication ability in these cultivars and study of different BAP concentration effect on the shoot proliferation capacity under *in vitro* conditions was done.

Materials and Methods

Plant material

Three-year old selected *Rubus* cultivars – 'Tulameen' and 'Black Jewel' (*Rubus idaeus* L.), 'Black Satin' (*Rubus fruticosus* L.) and 'Tayberry' (*Rubus fruticosus* x *Rubus idaeus*) grown in the pot in outdoor conditions were used as a material for shoot collection. Nodal segments with one axillar or apical buds were collected in February and used as primary explants. Explants were surface-sterilized by agitation for 2 min in 70% ethanol and for 6 min in 0.1% mercuric chloride ($HgCl_2$) with few drops of Tween 20. After this, explants were rinsed 3 times for 15 min with sterile distilled water.

In vitro culture establishment and shoot multiplication

For shoot initiation, Murashige & Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 30 $g*L^{-1}$ sucrose, 8 $g*L^{-1}$ phytoagar, 0.5 $mg*L^{-1}$ 6-benzylaminopurine (BAP) and 0.2 $mg*L^{-1}$ indole-3-butyric acid (IBA) was used. Cefotaxime in concentration 150 $mg*L^{-1}$ was added to the medium to minimize contamination. Media were adjusted to pH 5.6 before autoclaving (20 min at 1 $kg*cm^3$, 121 °C). After sterilization, the explants were placed on Petri dishes (90 mm diameter, 4 explants on one Petri dish). After 4 weeks of cultivation, the initiated shoots were separated and transferred on fresh MS medium supplemented with 30 $g*L^{-1}$ sucrose, 8 $g*L^{-1}$ phytoagar, 1 $mg*L^{-1}$ BAP and 0.2 $mg*L^{-1}$ IBA for shoot multiplication. Polypropylene plastic containers (Combiness) were used for cultivation, in each 5 shoots were planted, totally 30 explants per each cultivar.

The number of shoots per explant was evaluated over 4 subcultures (labeled as I-IV) on the same multiplication medium, while the length of one subculture was 4 weeks. Multiplication coefficient (Q) was calculated as number of shoots/number of explants ratio and resulted as the total average number of shoots per explant.

Influence of BAP concentrations on shoot proliferation

For evaluation of different BAP concentration influence on shoot proliferation, cultivars 'Black Satin' and 'Black Jewel' were used. Explants were isolated from *in vitro* cultures grown on MS medium with 1 mg*L^{-1} BAP and 0.2 mg*L^{-1} IBA. The culture medium was MS medium supplemented with 30 g*L^{-1} sucrose, 8 g*L^{-1} phytoagar, auxin IBA at 0.2 mg*L^{-1}, and cytokinin BAP in four different concentrations (0.5 – 2 mg*L^{-1}). Media were labeled as variant 1 (V_1) – variant 4 (V_4):
V_1) 0.5 mg*L^{-1} BAP + 0.2 mg*L^{-1} IBA
V_2) 1 mg*L^{-1} BAP + 0.2 mg*L^{-1} IBA
V_3) 1.5 mg*L^{-1} BAP + 0.2 mg*L^{-1} IBA
V_4) 2 mg*L^{-1} BAP + 0.2 mg*L^{-1} IBA

Into each plastic vessel 5 shoots was planted, totally of 30 explants of each variety was tested. The number of shoots per explant was evaluated over 3 subcultures, while the length of one subculture was 4 weeks.

For root induction, the isolated shoots 2.0 ± 0.5 cm long were cultured on MS medium supplemented with 30 g*L^{-1} sucrose, 8 g*L^{-1} phytoagar and 1 mg*L^{-1} IBA. All cultures were maintained in a growth chamber at 22±2 °C using a 16 h light/8 h dark photoperiod. Light was supplied using white fluorescent lamps at an intensity of 50 µM$m^{-2}s^{-1}$.

Acclimatization of plants under *ex vitro* conditions

After 6 weeks of cultivation on rooting medium, shoots were removed from vessels and were carefully washed under running water in order to remove the agar and transferred *ex vitro* to covered plastic pots with soil substrate. Plantlets were sprayed with water for first weeks. After 3 weeks of growth, culture vessels have been gradually opened to achieve acclimatization of the plants to outdoor conditions.

Statistical analysis

The results from individual experiments were statistically evaluated by using software Statgraphic Centurion XV analysis of variance (ANOVA). Data were analyzed by Tukey's HSD test.

Results

MS medium supplemented with 0.5 mg*L^{-1} BAP and 0.2 mg*L^{-1} IBA was proper for *in vitro* culture establishment of tested *Rubus* cultivars. Almost 100% of explants initiated shoots on this medium. However, approximately 50% of cultures contaminated in spite of the fact that strong sterilization agent ($HgCl_2$) was used for

explant sterilization. Longer sterilization (8 min) with 0.1% $HgCl_2$ caused damage and necrosis of explants (data not shown). Because of these findings, antibiotic cefotaxime was added to the culture medium what resulted in avoiding of contamination.

Shoot multiplication ability as influenced by cultivar and subculture

Shoot multiplication took place on medium with 1 mg*L^{-1} BAP and 0.2 mg*L^{-1} IBA. Evaluation of the shoot number/explant in each cultivar after 4 subcultures showed that shoot proliferation ability in individual *Rubus* spp. cultivars was different. The results of testing the ability of shoot multiplication by individual cultivars are summarized in Table 1.

Table 1. Average number of shoots per explant for individual cultivars during 4 subcultures expressed as multiplication coefficient (Q)

Tabuľka 1. Priemerný počet výhonkov na explantát pri jednotlivých kultivaroch počas 4 subkultivácií vyjadrený ako multiplikačný koeficient (Q)

Cultivar	Subculture	Number of explants	Number of shoots	Q	Average Q
'Black Jewel'	I	30	66	2.2	2.34
	II	30	65	2.16	
	III	30	50	1.66	
	IV	30	101	3.36	
'Tayberry'	I	30	53	1.76	2.9
	II	30	76	2.53	
	III	30	113	3.76	
	IV	30	107	3.56	
'Tulameen'	I	30	72	2.4	3.59
	II	30	130	4.33	
	III	30	116	3.86	
	IV	30	114	3.8	
'Black Satin'	I	30	142	4.73	4.08
	II	30	85	2.83	
	III	30	133	4.43	
	IV	30	130	4.33	

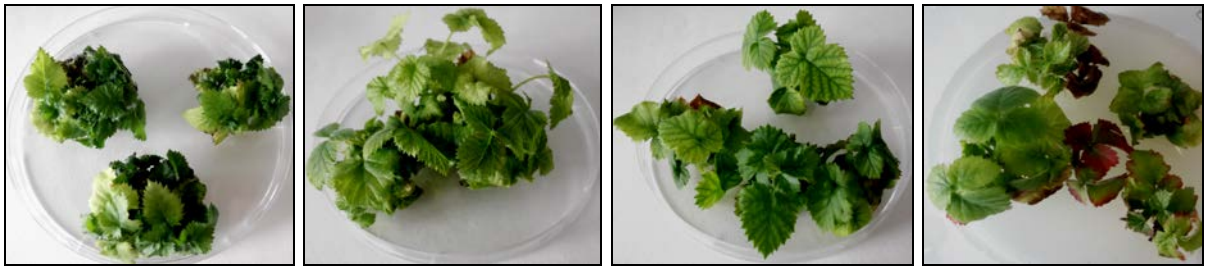

Figure 1. Shoot multiplication by individual *Rubus* spp. cultivars propagated under *in vitro* conditions. From left to right: 'Black Satin', 'Tulameen', 'Tayberry', 'Black Jewel'

Obrázok 1. Multiplikácia výhonkov jednotlivých kultivarov *Rubus* spp.kultivovaných *in vitro*. Zľava do prava: 'Black Satin', 'Tulameen', 'Tayberry', 'Black Jewel'

Table 1 shows that the highest ability of shoot multiplication was recorded by 'Black Satin', where the average number of shoots per explant was Q = 4.08. The obtained shoots were vital in dark green colour. For 'Tulameen', medium intensity of shoot proliferation (Q = 3.59) was observed. The formed shoots were also vital, but rather light green than dark green. 'Tayberry' showed lower multiplication coefficient (Q = 2.9) under these culture conditions. The shoots were tiny, pale green and necrosis was observed around the leaf edges. The lowest multiplication rate was recorded at 'Black Jewel' (Q = 2.34), which also exhibited poor recovery during subcultures. Its shoots and leaves were small, light green and often underwent necrosis (Figure 1). The statistical evaluation of cultivar shoot proliferation ability showed that between cultivars 'Black Satin' and 'Tulameen' was not statistically significant difference in the shoot multiplication, similarly as between 'Tayberry' and 'Black Jewel'. A statistically significant difference was found between the two groups of cultivars ('Black Satin' and 'Tulameen' versus 'Tayberry' a 'Black Jewel'), as shown in Table 2.

Table 2. Statistical evaluation of the differences in the average number of shoots per explant among tested cultivars (Tukey's HSD test)

Tabuľka 2. Štatistické hodnotenie rozdielov v priemernom počte výhonkov na explantát medzi testovanými kultivarmi (Tukey-ov HSD test)

Cultivar	Average number of shoots per explant	Homogenous groups	
		1	2
'Black Jewel'	2.5636	****	
'Tayberry'	2.9083	****	
'Tulameen'	3.6000		****
'Black Satin'	4.0833		****

By evaluation of total average number of shoots/explant during single subcultures, it was found that the number of shoots was rising with number of subcultures (Table 3). The highest average number of shoots per explant was obtained during IV

subculture. This could imply certain adaptation of plants tissue on *in vitro* conditions. However, statistical analyses did not show any significant differences between I and II subculture, as well as between the III and IV subculture. A statistically significant difference was found between the two groups of subcultures (I and II versus III and IV), as shown in Table 4.

Table 3. Average number of shoots per explant during individual subcultures

Tabuľka 3. Priemerný počet výhonkov na explantát počas jednotlivých subkultivácií

Subculture	Cultivar				Average number of shoots per explant
	'Black Jewel'	'Tayberry'	'Tulameen'	'Black Satin'	
I	2.20	1.76	2.40	4.73	2.77
II	2.16	2.53	4.33	2.83	2.96
III	1.66	3.76	3.86	4.43	3.43
IV	3.36	3.56	3.80	4.33	3.76

Table 4. Statistical evaluation of the differences in the average number of shoots per explant between individual subcultures (Tukey's HSD test)

Tabuľka 4. Štatistické hodnotenie rozdielov v priemernom počte výhonkov na explantát medzi jednotlivými subkultiváciami (Tukey-ov HSD test)

Subculture	Average number of shoots per explant	Homogenous groups	
		1	2
I	2.7750	****	
II	2.9666	****	
III	3.4300		****
IV	3.7666		****

Shoot multiplication ability as influenced by BAP concentrations

Plant growth regulators added in the culture medium are one of the key factors that contribute to the growth induction and multiplication of the shoots. Therefore several BAP concentrations (0.5 mg*L^{-1} - 2 mg*L^{-1}) in combination with 0.2 mg*L^{-1} IBA were tested in order to determine optimal conditions for shoot multiplication. The results of testing various BAP concentrations are shown in Table 5.

Table 5. Tested variants of media with various concentrations of cytokinin BAP supplemented with 0.2 mg*L IBA (V_1-V_4) and their impact on number of shoots per explant

Tabuľka 5. Testované varianty médií s rôznymi koncetráciami cytokinínu BAP doplnené s 0.2 mg*L^{-1} IBA (V_1-V_4) a ich vplyv na počet výhonkov na explantát

Medium type	Number of explants	Number of shoots per explant		Average number of shoots per explant
		'Black Satin'	'Black Jewel'	
V_1	30	4.10	2.38	3.24
V_2	30	5.29	4.15	4.72
V_3	30	5.30	4.48	4.89
V_4	30	5.86	4.40	5.13

Results shown in Table 5 confirmed the positive effects of increased BAP concentrations on shoot multiplication in different cultivars. The highest number of shoots per explant was recorded in 'Black Satin' at 2 mg*L^{-1} BAP (V_4) and in 'Black Jewel' at 1.5 - 2 mg*L^{-1} BAP (V_3 and V_4). The lowest average number of shoots occurred on V_1 medium, where the concentration of BAP was 0.5 mg*L^{-1}. The statistical evaluation of these results is in the Table 6.

Table 6. Influence of different concentrations of BAP on shoot proliferation evaluated by Tukey's HSD test

Tabuľka 6. Vplyv rôznych koncentrácií BAP na proliferáciu výhonkov hodnotený Tukey-ovým HSD testom

BAP concentration (mg*L^{-1})	Average number of shoots per explant	Homogenous groups	
		1	2
0.5	3.4666		****
1.0	4.7333	****	
1.5	4.8833	****	
2.0	5.2545	****	

Data in Table 6 clearly showed that there is statistically significant difference in the intensity of the shoot formation on the medium with 0.5 mg*L^{-1} BAP and other tested BAP concentrations. But, no significant differences were observed between 1; 1.5 and 2 mg*L^{-1} BAP concentrations. The results indicate that increased concentrations of BAP (1-2 mg*L^{-1}) have equally positive impact on shoot formation. Comparing the number of shoots produced by each cultivar, a statistically significant difference in the ability of the shoot multiplication between 'Black Satin' and 'Black Jewel' was

confirmed. The total average number of shoots per explant obtained by ‘Black Satin’ was 4.9 and by ‘Black Jewel’ 3.84 (Table 7).

Table 7. Genotype influence on shoot multiplication evaluated by Tukey's HSD test

Tabuľka 7. Vplyv genotypu na multiplikáciun výhonkov hodnotený Tukey-ovým HSD testom

Cultivar	Average number of shoots per explant	Homogenous groups	
		1	2
‘Black Jewel’	3.8434	****	
‘Black Satin’	4.9066		****

In vitro rooting and acclimatization

Rooting of isolated shoots was achieved on the rooting medium containing 1 mg*L^{-1} IBA. First *in vitro* roots were observed after 6 weeks of incubation. Rooting was successful at approximately 80-100%. After developing a sufficient number of roots, plantlets were transferred *ex vitro* to the pots with soil substrate. Acclimatization was successful at approximately 50%.

Figure 2. *In vitro* root formation in ‘Black Satin’ (left), acclimatization of ‘Black Satin’ plantlets (right)

Obrázok 2. *In vitro* zakoreňovanie výhonkov pri ‘Black Satin’ (vľavo), aklimatizácia rastlín ‘Black Satin’ (vpravo)

Discussion

Rubus genus is an economically important group of small fruit, including raspberries, blackberries and dewberries (Jennings et al., 1991). Micropropagation can partly fulfill the increasing demand for these fruits on the market and food industry. That is why many experiments focus on optimizing each step of this process in order to develop an effective multiplication system for *Rubus* spp. explants.

Origin of primary explants belongs to the important factors by establishing the *in vitro* cultures. Cultivation of nodal segments with dormant apical and axillar bud as primary explants resulted in almost 100% successful shoot initiation. These results are consistent with observations of other authors. Stoevska et al. (1995) used dormants buds for establishing *in vitro* cultures of two *Rubus idaeus* cultivars 'Shopska Alena' and 'Samodiva'. From all explants, 85-90% developed to shoots. Similarly, Ružić and Lazić (2006) used dormant buds for establishing *in vitro* cultures of *Rubus fruticosus* ('Čačanska Bestrna') and *Ribes nigrum* ('Čačanska Crna'), although they achieved lower percentage of shoot initiation.

The obtained results confirmed that genotype affects regeneration ability under *in vitro* conditions. In this experiment, *Rubus fruticosus* cv. 'Black Satin' showed to have higher shoot multiplication ability than *Rubus idaeus* cultivars. Differences were recorded also between two *R. idaeus* cultivars, when 'Tulameen' produced higher average number of shoots per explant than 'Black Jewel'. The effect of genotype has already been reported within two *R. idaeus* cultivars 'Gradina' and 'Willamette' by Gonzales et al. (2000). In this study, higher regeneration and multiplication ability in *R. fruticosus* compared to *R. idaeus* was also confirmed. Similar results were observed also by Graham et al. (1997), who compared several raspberry cultivars to determine their *in vitro* regeneration ability.

The experiment focused on finding optimal concentration of BAP for shoot multiplication showed that higher BAP concentrations (1-2 $mg*L^{-1}$) had more positive impact on shoot formation than lower concentration (0.5 $mg*L^{-1}$). These results are consistent with most of published protocols for *Rubus* spp. micropropagation. Donnelly et al. (1980) used 1 $mg*L^{-1}$ BAP with 0.1 $mg*L^{-1}$ IBA for *in vitro* propagation of *R. occidentalis, R. idaeus* and *R. loganobaccus*. Bobrowski et al. (1996) reported that 1-2 $mg*L^{-1}$ BAP is sufficient for *R. fruticosus* micropropagation. On the contrary, Wu et al. (2009) achieved improved shoot formation in several *Rubus* species using 3 $mg*L^{-1}$ BAP compared to 1 $mg*L^{-1}$. However, he also reported signs of vitrification among several cultivars when cultivated at higher BAP concentration. It is known that too high concentrations of cytokinines can inhibit the shoot elongation. In this experiment, the obtained shoots were characterized by good elongation growth therefore the conclusion can be made that concentrations of 1-2 $mg*L^{-1}$ BAP are sufficient and suitable for efficient shoot proliferation and vigorous shoot growth of searched *Rubus* cultivars.

Conclusion

This study describes protocols for micropropagation of four *Rubus* spp. cultivars. It was found out that genotype and subcultivation significantly influence regeneration and multiplication ability under *in vitro* conditions. The highest number of shoots per explant was observed in 'Black Satin'. The experiments focused on influence of different BAP concentration on shoot proliferation showed that the best results were achieved with 2 $mg*L^{-1}$ BAP in cv. 'Black Satin'. Explants were successfully rooted and acclimatized under *ex vitro* conditions.

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