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IN VITRO MULTIPLICATION AND ROOTING OF BLACK RASPBERRY CV. CUMBERLAND (*RUBUS OCCIDENTALIS* L.)

SUMMARY

The aim of this study is to develop an efficient *in vitro* multiplication and rooting system for black raspberry (*Rubus occidentalis* L.).

The interest of selectors and producers in this fruit is constantly growing due to their positive impact on human health on the one hand, and also the taste which is more intense than the traditional red raspberries. They combine the juiciness of blackberries with the texture and sweetness of red raspberries. The American black raspberry cultivar Cumberland was used to fulfil this purpose.

An eight variants system was developed to test the effect of cytokinins BAP (6-Benzylaminopurine) and TDZ (Thidiazuron) at different concentrations (0.2, 0.1, 0.05 mg/l) on the propagation process based on MS (Murashige and Skoog, 1962) medium. The effect of casein hydrolysate (500 mg/l) on the micropropagation process added to MS 8 medium was also studied.

The highest multiplication coefficient was recorded in variant MS 2 with 6.4 shoots per explant in the first passage, and the best result in terms of average shoot length was recorded in MS1- 3.21 cm.

To stimulate the process of rhizogenesis under *in vitro* conditions, auxins - IBA, IAA, NAA (indole-3-butyric acid, indole-3-acetic acid, naphthaleneacetic acid) were tested at concentrations of 0.1 and 0.2 mg/l, addition of myo-inositol (500 mg/l) based on MS medium with reduced salt concentration. The best result was reported for R 2 (0.2 mg/l IAA), R 3 (0.2 mg/l NAA), R 4 (0.1 mg/l IBA) and hormone-free medium R 7 (control).

Keywords: *Rubus occidentalis* L., micropropagation, tissue culture, myo-inositol, casein hydrolisat

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Abbreviations: IAA (Indole-3-acetic acid), IBA (indole-3-butyric acid), NAA – naphthaleneacetic acid CH - casein hydrolisate, MI – myo-inositol, BAP –6-benzylaminopurine, TDZ – thidiazuron

INTRODUCTION

Rubus occidentalis L., is a plant species belonging to the family *Rosaceae* genus *Rubus*, which is one of the most widespread in the plant kingdom. The interest in its fruits is due to its rich content of antioxidants, favorably affecting physiological processes in the human organism (Alappat and Alappat, 2020; Pap *et al.,* 2021; Ponder *et al.,* 2021; Toshima *et al.,* 2021; Aguilera and Toledo, 2022; Petruskevicius *et al.* 2023).

In addition, *Rubus occidentalis* L., is also of interest for selection and is commonly used as a donor of resistance to root rot (genus *Phytophtora*) and the large raspberry aphid (*Amphorophora idaei* Borh), a vector of raspberry mosaic virus in Europe (Drain 1956; Ellis *et al.,* 1992; Dossett *et al.,* 2008; Dossett and Finn 2010).

The first report of clonal micropropagation of *Rubus occidentalis* L. dates back to 1978 and was made by Harper (1978). Since then, many papers on multiplication of members of *Rubus* have been published, overcoming some of the problems associated with them (Anderson, 1980; Donnelly *et al.,* 1980; Upadyshev and Vysotsky 1995; Upadyshev and Guskov, 1996; Georgieva *et al.,* 2016; Georgieva *et al.,* 2020; Raeva-Bogoslovskaya *et al.,* 2021; Yosefi *et al.,* 2022; Topçu, 2022).

Phytohormones are particularly important for the coordination of growth and development processes in plant organisms. The main role of cytokinins is related to the stimulation of cell division, overcoming apical dominance, in addition, they are involved in the regulation, growth and differentiation of cells (Sharma, 2017; Ojol *et al.,* 2018; Kieber *et al.,* 2018; Petrasek *et al.,* 2019; Sharma and Zheng, 2019; Xue *et al.,* 2020; Konka *et al.,* 2021; Raspor *et al.,* 2021; Šmeringai *et al.,* 2023; Pokimica *et al.,* 2024). The most commonly used cytokinin with universal action in the micropropagation process in raspberry is BAP., in doses of 0.2 mg/l - 3 mg/l (Hunková *et al.,* 2016; Raeva-Bogoslovskaya *et al.,* 2021; Yosefi *et al.,* 2022; Topçu, 2022). Plant hormones do not act isolated in the plant organism, but are linked in complex interactions to each other. Cytokinins are sometimes used in combination with auxins (IAA, IBA, NAA), at a lower concentration than cytokinin, to increase their influence in *in vitro* propagation in genus *Rubus*. A balanced auxin:cytokinin ratio has important implications for the development of optimized protocols in genus *Rubus* representatives.

The use of synthetic non-purine compounds that possess potent cytokinin activity such as thidiazuron (TDZ) is increasingly required in tissue cultivation (Huetteman and Preece, 1993; Zhou *et al.,* 1994; Dai *et al.,* 2006).

Application of low doses of auxins during *in vitro* rooting of genus Rubus representatives stimulates the process of rhizogenesis and positively affects the

quality of the resulting plants. In the scientific literature, there are reports of enhanced root formation after their addition to the nutrient medium (Upadyshev and Visotsky 1995; Upadyshev and Guskov 1996; Georgieva and Kondakova 2009; Georgieva *et al.,* 2016; Hunková *et al.,* 2016; Georgieva *et al.,* 2020; Raeva-Bogoslovskaya *et al.,* 2021; Yosefi *et al.,* 2022; Topçu 2022).

The aim of the present study is to investigate the influence of the type and concentration of the growth regulators BAP, TDZ and the organic additives casein hydrolisate and myo-inositol on the *in vitro* cultivation, multiplication and rooting of the cultivar Cumberland (*Rubus occidentalis* L.).

MATERIAL AND METHODS

Cumberland cultivar, which is poorly distributed and studied in Bulgaria, was selected as the subject of this experiment. The cultivar is included in the Corvallis genebank (National Clonal Germplasm Repository-Corvallis (USDA-ARS NCGR) listed as 553739 (Dossette et al., 2012). Cumberland is an American cultivar characterized by weak growth, stalked, with thin shoots that lodge. The shoots are dark red coloured, covered with a grey waxy plaque that decreases towards the top. They have spines at their base which decrease towards the top.

Sterilization of plant material and introduction into in vitro culture

For *in vitro* culture, initial explants of defoliated two-bud green cuttings 1.5-2 cm long and the apical buds from *in vivo* plants of cv Cumberland were used. Microcuttings are isolated from the middle part of annual shoots. MS (Murashige and Skoog, 1962) medium enriched with BAP 0.5 mg/l and IBA 0.01mg/l and pH 5.6 adjusted before autoclaving was used for initial introduction of raspberry cuttings. A standard sterilization system was used (Georgieva *et al.,* 2016).

Sterilization of nutrient medium

After adjusting the pH of the medium to 5.6, all media were autoclaved at 1.2 kPa pressure and 121 °C for 20 min.

Microcuttings and apical buds of Cumberland cultivar introduced into *in vitro* culture were raised in growth cabins under controlled conditions-temperature was 22±25 °C, photoperiod 16/8 days/night and illumination 2000-3000 lx. The duration of subculturing is 30 days. MS basal nutrient medium with varying concentrations of cytokinins (BAP and TDZ - 0.2 mg/l; 0.1 mg/l; 0.05 mg/l), auxins (IAA, IBA, NAA - 0.1 mg/l; 0.2 mg/l) and organic additives (myo-inositol, casein hydrolysate - 500 mg/l) were used for *in vitro* multiplication and rooting (Table 1 and 2).

After 30 days of cultivation, plants were aseptically separated and transferred to fresh nutrient medium.

Table 1. Variants for multiplication of black raspberry based on MS basal nutrient medium

Growth regulators and organic additive	Variants							
	MS 1	MS 2	MS 3	MS 4	MS 5	MS 6	MS 7	MS 8
BAP mg/l	-	0.7	0.5	0.2				0.5
TDZ mg/l	-				0.05	0.1	0.2	
IBA mg/l			0.01					0.01
CH mg/l								500

Table 2. Variants for *in vitro* rooting of black raspberry based on MS basal nutrient medium

Growth regulators and organic additive	Variants							
	R 1	R 2	R 3	R 4	R 5	R 6	R 7	R 8
IBA mg/l	0.2			0.1			-	
IAA mg/l		0.2			0.1		-	
NAA mg/l			0.2			0.1	-	
myo-inositol (MI mg/l)								500

Adaptation

Adaptation of Cumberland (*Rubus occidentalis* L.) *in vitro* plants under non-sterile conditions was carried out under polyethylene tunnels, under greenhouse conditions, for 30 days. A peat was used in combination with sand (1:1) and pH 5.5-6.5. Successful transfer from *in vitro* to *ex vitro* conditions depends on their ability to grow rapidly and the provision of suitable phytosanitary conditions. After a two-week growing period, the plants are transferred to larger containers for further growth.

For the statistical processing of the data, analysis of variance ANOVA was used.

RESULTS AND DISCUSSION

The data were statistically significant ($P<0.05$) and indicated that the multiplication coefficient was affected by the type, concentration and combination of growth regulators or organic supplement applied in the nutrient medium (Figure 1). All applied concentrations of purine cytokinin BAP resulted in enhanced development of multiple newly formed shoots. Statistically

significant differences were observed between BAP-containing nutrient medium (MS 2) and those with TDZ (MS 5, MS 6 and MS 7) (Figure 1).

The stimulating effect of BAP for new shoot formation has been reported for other species of genus *Rubus* (Turovskaya and Strishna, 1900; Hoepfner and Nestby, 1991; Stoevska *et al.,* 1995; Ferradini et al., 1997; Ružić and Lazić 2004; Velchev, 2004; Georgieva *et al.,* 2020; Clapa *et al.,* 2021; Raeva-Bogoslovskaya, 2021). The use of higher levels of BAP (0.7 mg/l) had a positive effect on the multiplication potential of the studied cultivar, as the highest average value of this indicator was recorded on MS 2 medium - 4.90 pcs/explant. The lowest average value of the studied indicator was recorded on hormone-free medium (MS 1) - 1.34 pcs./explant (Figure 1). In variant MS 3, no improvement in multiplication potential was recorded (1.66 pcs./explant).

In his study Anderson, (1980) reported that red and black raspberry cultivars respond differently to the concentrations and combinations of growth regulators used.

Donnelly *et al.,* (1980) introduced apical meristem of Munger genotype (*Rubus occidentalis* L.) to MS basal nutrient medium supplemented with 1 mg/l IBA and 0.1 mg/l BAP. For shoot proliferation, the same growth regulators were used but at different concentrations, 1 mg/l BAP and 0.1 mg/l IBA. The most efficient rooting they achieve on the same basal medium enriched with 0.5-0.7 mg/l IBA.

Similar results to ours in terms of the multiplication coefficient were reported by Upadyshev and Visotsky (1995), who achieved 4.4 pc/ shoot on MS basal nutrient medium supplemented with 1 mg/l BAP in the cultivar Cumberland, noticing a definite seasonal dependence i.e. that it was highest in winter and spring.

Hunkova *et al.,* (2016) found that the combination of 1 mg/l BAP combined with 0.2 mg/l IBA gave the highest multiplication coefficient (3.59) in cultivar Tulameen.

Including synthetic cytokinin TDZ in raspberry micropropagation medium was dictated by its high cytokinin activity in fruit species, superior to classical hormones used in plant tissue culture (Huetteman and Preece, 1993; Zhou *et al.,* 1994; Dai *et al.,* 2006). Proliferation under *in vitro* conditions of difficult-to-reproduce species has been successful by its integration into multiplication medium (Huetteman and Preece, 1993). The results of our experiment show its inhibitory effect on the multiplication coefficient of Cumberland plants. *In vitro* cultures initiated on all three media supplemented with different concentrations of TDZ (0.05 mg/l; 0.1 mg/l; 0.2 mg/l) were hyperhydrated and fragile when subcultured on fresh medium.

A similar observation has been reported by Huetteman and Preece, (1993) and Gaspar *et al.*, (1996) who noticed that hyperhydration and insufficient elongation of cultures was observed with prolonged subculturing of microshoots on TDZ-enriched medium. The reported values of the multiplication coefficient

were no different from each other on medium enriched with different levels of TDZ (MS 5, MS 6 and MS 7).

The lowest value was recorded on the third passage (1.17 pcs/explant) of MS 5 medium enriched with the lowest concentration of TDZ (0.05 mg/l). Statistically proven differences of the studied indicator were noted on medium MS 2 and MS 3 compared to MS 4 and MS 8. Only variant MS 2 was mathematically different from variants MS 5, MS 6 and MS 7. Comparing different types of cytokinins, it can be concluded that BAP is more suitable than TDZ for accelerated *in vitro* multiplication of Cumberland cultivar, considering the quality of plants obtained.

The stimulatory effect of subcultivations was also reported by Donnelly *et al.,* (1980), who observed that as passage duration increased, so did the multiplication coefficient.

The addition of different types and combinations of exogenous growth regulators (auxins, cytokinins and gibberellins) to the basal nutrient medium stimulates the multiplication potential. Comparing the results of our previous studies with wild red raspberry (*R. idaeus* L.) we obtained the highest average number of shoots (6.8) on MS medium enriched with 0.1 mg/l IBA, 0.3 mg/l BAP and 0.1 mg/l GA3 (Georgieva *et al.,* 2016).

Interesting results were obtained on the MS 8 nutrient medium. The applied combination auxin (0.01 mg/l IBA) + cytokinin (BAP 0.5 mg/l) + (casein hydrolysate 500 mg/l) had a positive influence on the average value of the multiplication potential of the cultivar Cumberland, which was 3.11 pcs./explant. *In vitro* plants of this variant of the scientific experiment are characterized by dark green, large leaves, anthocyanin-colored stems, equal growth and high vigor. Increased leaf area under *in vitro* conditions will lead to more intense photosynthesis and transpiration in the field, which will be a prerequisite for strong vegetative growth.

Adapted plants are characterised by anthocyanin-coloured upper leaves, a large number of spines on the stem, intense growth and large leaf. Increased leaf area under *in vitro* conditions will lead to more intense photosynthesis and transpiration in the field, which will be a prerequisite for strong vegetative growth.

Adapted plants are distinguished by anthocyanin-coloured upper leaves, a large number of spines on the stem, strong growth and large leaf.

In the present experiment, an analogous combination of auxin-cytokinin levels in the nutrient medium was included, only without the participation of casein hydrolysate, on which a significantly lower average value of the multiplication coefficient, 1.66 pc/explant, was recorded, which could be taken as undeniable proof of the stimulatory role of organic additives on morphogenesis of the Cumberland cultivar.

In the course of the experimental work, steadily multiplying *in vitro* cultures of the cultivar Cumberland were initiated by reporting root induction in some of the variants tested with no or minimal (0.01 mg/l IBA) exogenous auxin

application (MS 1, MS 4, MS 8), which is likely due to the presence of high endogenous auxin levels in the plants, a trend that persisted in all five subcultivations. Similar results to ours for initiation of root growth on an accelerated multiplication medium enriched with the same plant hormones but at higher concentrations (1 mg/l BAP and 1 mg/l IBA) were also reported by Donnelly *et al.,* (1980).

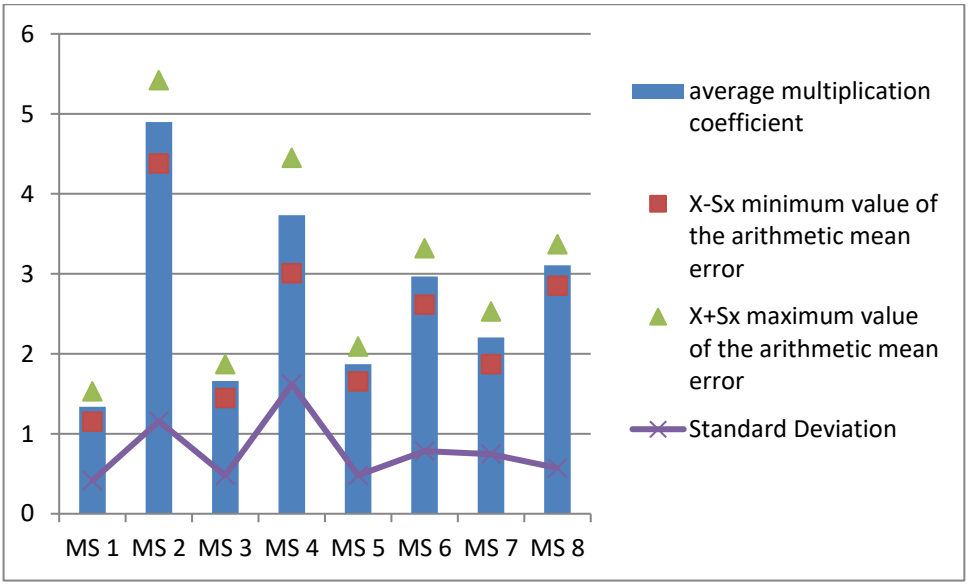
The values of the average shoot length index ranged from 1.30±0.11 cm (MS 5 containing 0.05 mg/l TDZ) to 2.78±0.21 cm on MS 1 (hormone-free medium), which is probably due to the inhibitory effect of this cytokinin on shoot height (Figure 2).

There was a trend towards an increase in the number of newly formed shoots and a decrease in shoot length without statistical significance ($p>0.05$) as BAP concentration increased (Table 3, Figure 2, 3).

In contrast to our results regarding shoot length are those reported by Upadyshev and Vysotsky (1995), on MS basal nutrient medium enriched with a higher dose of BAP (1 mg/L), on which a significantly greater height of newly formed shoots was also reported, 4.53 cm in the Cumberland cultivar.

Table 3. Multiplication coefficient by passages of the basal MS nutrient medium of the tested variants

Passages	**Variants**							
	MS 1	MS 2	MS 3	MS 4	MS 5	MS 6	MS 7	MS 8
I	1.00	6.40	1.21	6.00	1.92	4.00	1.73	2.50
II	2.00	4.67	1.26	4.25	2.17	2.67	2.17	3.20
III	1.25	3.67	2.38	4.08	1.17	2.33	1.75	3.08
IV	1.44	4.00	1.83	2.33	2.42	2.25	3.50	2.75
V	1.00	5.75	1.62	2.00	1.67	3.58	1.87	4.00
(SE)	0.19	0.52	0.21	0.72	0.22	0.35	0.33	0.26
(SD)	0.41	1.16	0.48	1.62	0.48	0.78	0.75	0.57
(P<0.05)	e	a	e	b	de	bcd	cde	bc



c MS 1 MS 2 MS 3 MS 4 MS 5 MS 6 MS 7 MS 8

(P<0.05) e a e b de bcd cde bc

Figure 1. Average multiplication coefficient of basal MS nutrient medium (± SE)

Table 4. Shoot length of Cumberland cultivar under different treatments grown on basal MS nutrient medium.

Passages	Variants							
	MS 1	MS 2	MS 3	MS 4	MS 5	MS 6	MS 7	MS 8
I	3.21	1.82	2.11	2.11	1.67	1.66	2.03	2.70
II	2.17	1.70	2.17	2.11	1.44	1.34	2.12	2.10
III	2.87	1.43	2.38	1.65	1.07	1.45	1.95	2.26
IV	2.46	2.38	2.11	2.38	1.16	1.30	1.43	1.86
V	3.21	2.13	1.71	2.31	1.15	1.22	1.68	2.18
(SE)	*0.21*	*0.17*	*0.11*	*0.13*	*0.11*	*0.08*	*0.13*	*0.14*

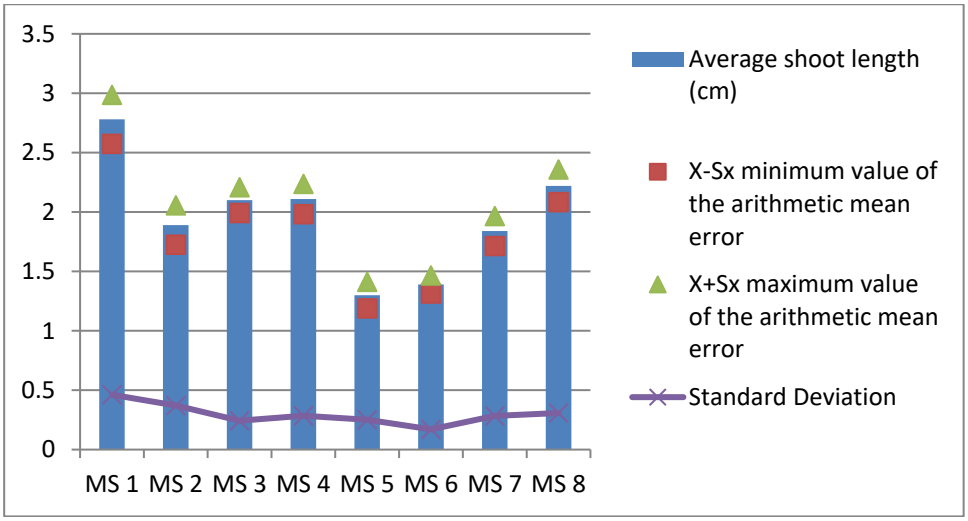


Figure 2. Average shoot length of the Cumberland cultivar on the tested nutrient medium variants (± SE)

Our observations on the rhizogenesis process in the cultivar Cumberland show that it is not affected by auxin concentration, proving that the level of endogenous auxins in the plant is sufficient to stimulate this process. The earliest root formation starts on the following nutrient medium: R 1, R 2, R 4, R 5, R 7. The rooting percentage was highest (100%) on the tested medium R 2 (0.2 mg/l IAA), R 3 (0.2 mg/l NAA), R 4 (0.1 mg/l IBA) and the hormone-free medium R 7 (Figure 4,7).

In the process of rhizogenesis, the observed differences are usually related to the quality of the roots obtained, which is of extreme importance for the *ex vitro* adaptation of plants in soil and their further development. The best results regarding the average root number was reported on nutrient medium R 4 (4.85 numbers) and R 1 (4.45 numbers), with good evidence of the differences ($P<0.01$) (Figure 5).

The induced roots without statistical significance ($P>0.05$) had the greatest average length of variant 1 (4.06 cm) and variant 7 (3.52 cm) (Figure 6). The addition of 500 mg/l myo-inositol resulted in a relatively low root formation rate of 25%, which determined its inhibitory role as a quantity in rhizogenesis of the Cumberland cultivar. The results obtained give us the reason to recommend the use of nutrient medium R 4 (0.1 mg/l IBA) for rooting of cultivar Cumberland, a variant in which the highest average root number (4.85 pcs) was also recorded (Figure 5).

Similar results to ours regarding rhizogenesis were noticed by Upadyshev and Visotsky (1995), who reported 100% rooting of the fifth passage in the Cumberland cultivar on MS basal medium, only with a significantly higher IBA content of 1 mg/L. The researchers obtained the best results on root length on the third passage - 2.93 cm and the highest number of roots - 8.1 cm on the same

nutrient medium. The authors noticed that there was a definite correlation between shoot length and rooting ability: as shoot length increased from 1.5-3 cm, root formation increased by 2 to 3.5 times. In addition, the same authors observed a positive correlation between the rooting percentage and the number of subcultivations of the *in vitro* culture.

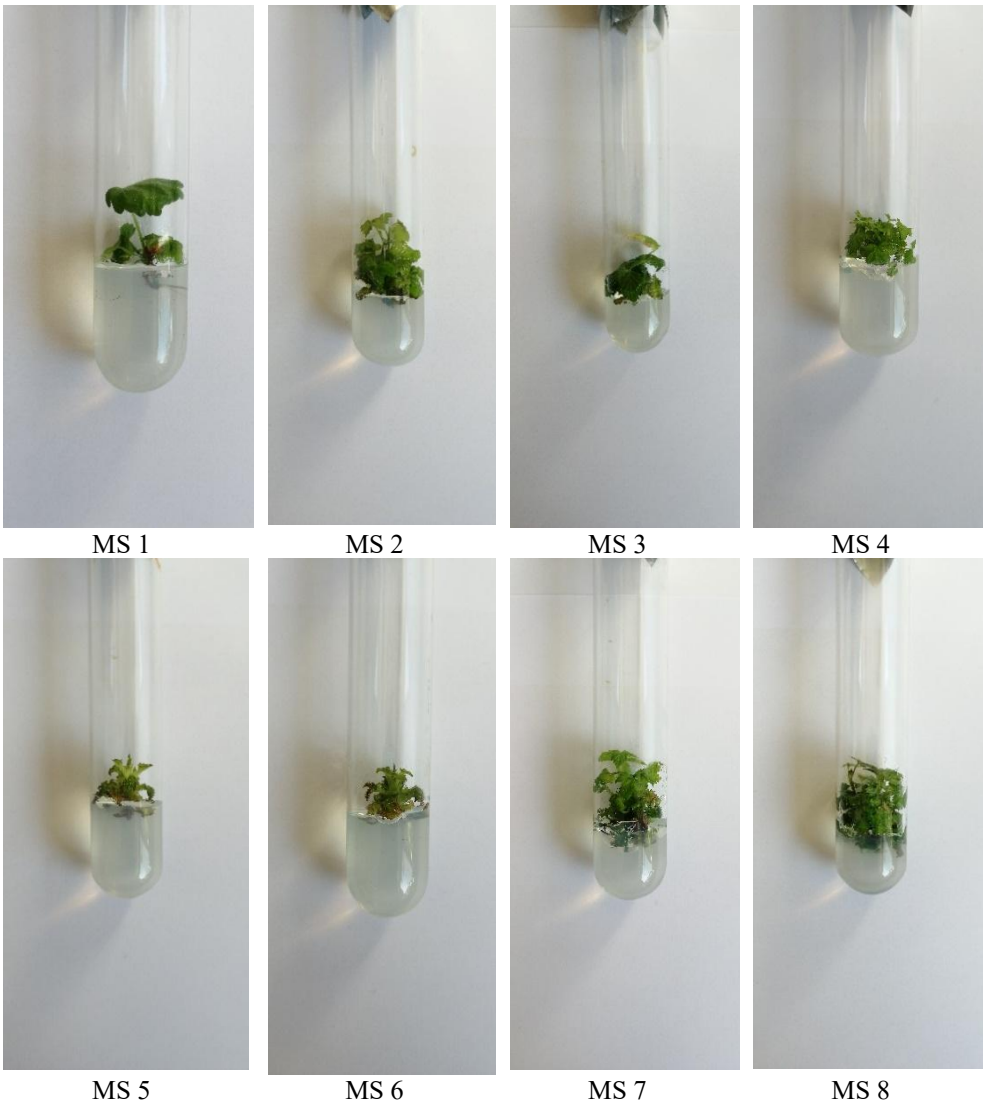


Figure 3. Multiplication coefficient of the cultivar Cumberland on different variants of nutrient medium

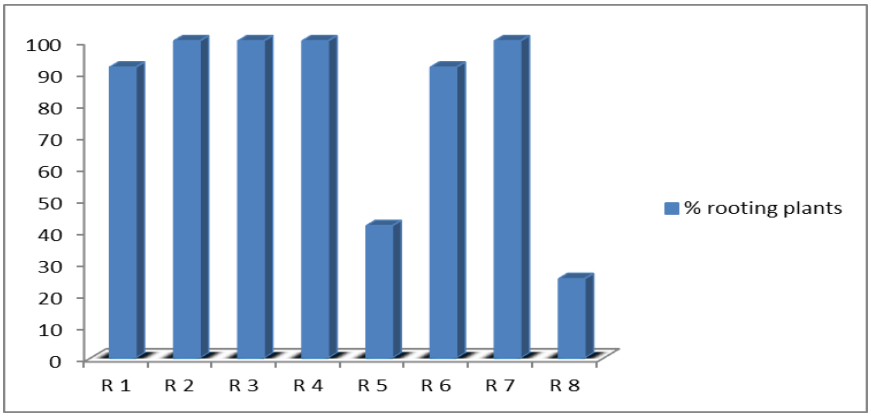


Figure 4. Rooting percentage of the cultivar Cumberland on different nutrient medium

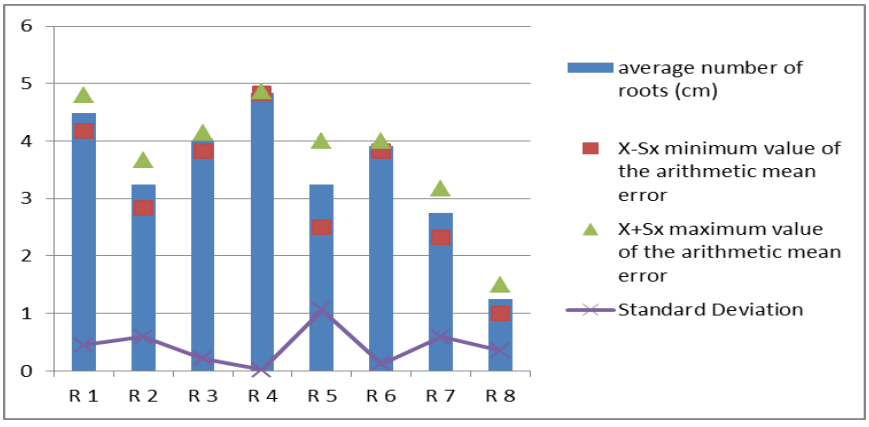


Figure 5. Average root number of Cumberland cultivar on different nutrient medium (±SE)

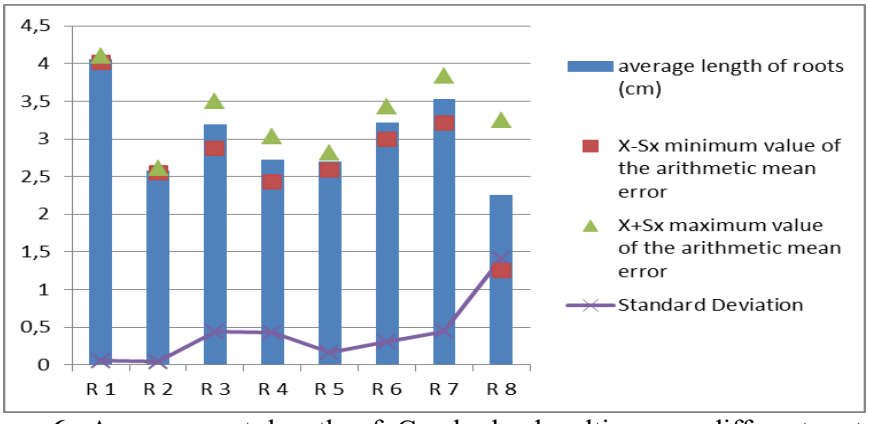


Figure 6. Average root length of Cumberland cultivar on different nutrient medium

Figure 7. *In vitro* rooting of Cumberland plants on different nutrient medium

Our results correlate with the studies of Upadyshev and Guskov (1996) on the rooting of black raspberry cv. Cumberland, which found that the combination of auxin (1 mg/l IBA) and chlorogenic acid (0.25 mg/l; 0.5 mg/l) had a strong positive effect on its rhizogenesis, resulting in 85% and 75% rooting ability.

Similar to our results for rooting percentage (80%-100%) were obtained by Hunkova *et al.,* (2016), but using higher doses of auxin (1 mg/l IBA) in the nutrient medium.

The developed system will be used in future studies as a basis for bioreactor cultivation of the cultivar Cumberland (*Rubus occidentalis* L.).

Adaptation

In vitro adaptation of *Rubus occidentalis* L. cv. Cumberland plants under laboratory conditions was unproblematic (Figure 8). Transfer from *in vitro* to *ex vitro* conditions was successful (90%), with no morphological changes observed and plants developing normally. The duration of adaptation was 30 days. The optimised conditions of container cultivation resulted in lively plants with equal growth and a well-developed root system.

Figure 8. Adaptation after day 14 of Cumberland plants *in vitro*

CONCLUSIONS

An efficient system for accelerated multiplication and rooting under *in vitro* conditions has been successfully developed for black raspberry (*Rubus occidentalis* L.) cv. Cumberland on MS basal nutrient medium (Murashige and Skoog, 1962).

Both apical and axillary buds can be used as explant sources.

The medium supplemented with an appropriate combination of plant hormones and organic additives led to the initiation of *in vitro* culture.

The addition of casein hydrolysate (500 mg/l) to MS variant 8 of multiplication medium showed positive effects on the multiplication rate and length of newly formed shoots. The results obtained from the rooting experiment showed that 500 mg/l myo-inositol had no stimulatory effect on rhizogenesis of cv Cumberland.

The application of low doses of exogenous auxins at the rooting stage resulted in efficient root formation, probably due to their high endogenous level in plants. The use of growth regulators at lower doses in perennial species guarantees the genetic stability of the resulting plant material obtained by applying biotechnological approaches to their multiplication.

The development of an efficient micropropagation protocol for black raspberry (*Rubus occidentalis* L.) cv. Cumberland will have practical application for enrichment, conservation, preservation and efficient use of the genetic material of this fruit species for inclusion in selection programs, material for tissue culture production in bioprocess engineering. The plants successfully adapted *in vitro* show no morphological changes and develop normally, they are equal in growth and ready for future observations and evaluations.

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