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A comparative study on raspberry cultivars in micropropagation

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Abstract

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The aim of the present study was to clarify the effectiveness of a simplified protocol for micropropagation of raspberry cultivars, such as Samodiva (control), Meeker, Willamette and the candidate cultivar of Magdalena (passed through DUS test) as prospective ones, which are suitable for cultivation in the mountain and hilly regions of Bulgaria. The effect of growth regulators BAP (0.5 mg l^{-1}) and IBA (0.01 mg l^{-1}) on proliferation capacity and shoot length over six passages was established. The highest multiplication potential 3.9 at the fifth passage and average length of the shoots 3.76 cm (fourth passage) were registered in the candidate cultivar of Magdalena. The best rhizogenic ability was recorded in Samodiva – 80.5%. These results show that genotype is the primary factor determining a high propagation and economically significant efficiency in this process. The application of simple medium for all studied genotypes is towards commercial propagation, comparing their growth characteristics and obtaining pre-base material for planting in a production nursery and further investigations.

Keywords: in vitro propagation; raspberries; growth regulators; proliferation capacity; root formation
Abbreviations: BAP – benzylaminopurine; IBA – indole-3-butyric acid; GA_3 – gibberellic acid; MS – basic nutrient media (Murashige&Skoog,1962)

Introduction

The cultivation of raspberries in Bulgaria, both in large industrial crops and in private yards, is increasing because of the suitable soil and climatic conditions. This tendency is due to the many years of tradition of cultivating that crops and demand for a variety of fresh fruits on the market, which determines the increasing interest of producers in growing new raspberry cultivars. One of the limiting factors for growing raspberry is the lack of certified (virus/pathogen free) planting material as a prerequisite for creating healthy, homogeneous and authentic raspberry plantations that provide high and stable yields.

Developing protocols for *in vitro* propagation of different raspberry cultivars is a guarantee for their more efficient inclusion in the production cycle. Plant tissue cultures find a widespread application in the accelerated production of raspberry planting material due to the ability of this method to provide a high percentage of pathogen-free planting material (Muster & Lance, 2002; Kondakova et al., 2005).

The most commonly used basal culture media in the micropropagation of raspberries are various modifications of Murashige & Skoog (1962); Linsmaier & Skoog (1965); Snir (1981); Sobczykiewicz (1987), and significantly less frequently those of Anderson (1980); James et al. (1980) and Avitia Garcia et al. (1985).

The most commonly used cytokinins for multiplication of raspberries is BAP (0.1–3.0 mg l^{-1}) alone or in combination with IBA (0.1–1.0 mg l^{-1}) and GA_3 (0–0.1 mg l^{-1}). Desjardines & Gosselin (1987) and Klokonos et al. (1987) reported that

MS basal culture medium supplemented with 0.5–1.0 mg l^{-1} BAP is the best for the proliferation of different raspberry cultivars. The multiplication capacity strongly depends on the type and concentration of cytokinin used, the duration of subcultivation, and last but not least on the genotype (Donnelly et al., 1980; Pyott & Convers, 1981; Isac & Popescu, 2009; Zaprianova & Ivanova, 2016; Zaprianova et al., 2018).

According to some authors (Sobczykiewicz, 1980; Vysotsky, 1984; Welander, 1985), the micropropagated raspberry plants can be rooted directly in a soil-peat mixture without stimulating of root formation *in vitro*. The enrichment of medium by activated carbon (Anderson, 1979; 1980) or 10 mM phloroglucinol (James et al., 1980; Sobczykiewicz, 1987) improves the root formation in raspberries.

The purpose of this study is to establish the effectiveness of a universal culture medium for *in vitro* propagation of raspberry cultivars by comparing their growth characteristics and obtaining of pre-basic material for planting in production fruit tree nurseries for further research.

Material and Methods

Source plant material

The scientific experiment was carried out in a laboratory for micropropagation of small-sized fruit species at RIMSA in Troyan. The study included the raspberry cultivars Samodiva as standard and the perspective candidate-cultivar Magdalena, Meeker and Willamette, which have a different origin (Table 1). These varieties are suitable for cultivation in the mountain and hilly regions of Bulgaria where they are well-provided with soil and atmospheric humidity. The selected genotypes are distinguished by proper shoot formation, moderate to strong growth, very good fruitfulness and average-sized to large-sized fruit (Leposavić et al., 2006; Petrović & Leposavić, 2011; Leposavić et al., 2015).

Explants were isolated from young, one-year shoots after a test for economically significant viruses (such as Raspberry leafspot, Raspberry leaf mottle, Rubus yellow net virus, Arabis mosaic virus, Raspberry ringspot virus, Tomato black ring virus) by a biological test on *Rubus occidentalis* and DAS ELISA serological method. The donor plants were cultivated in individual containers with a sterile soil substrate in insulating structures coated with a fine mesh to prevent further contamination by vectors (aphids and nematodes).

The mathematical processing was done using Lidanski statistical methods (1988).

Micropropagation

A standard sterilization system was applied by treating with 95% ethanol for 30 s and 5% bleach solution for 2.5 min, followed by 5X rinsing with sterile distilled water.

For all steps of micropropagation process MS nutritional medium was used, supplemented with different concentrations of growth regulators (Table 2). In the rooting stage, the salt concentration of the culture medium was reduced in half.

Table 2. Concentration of growth regulators in the culture media used for raspberry micropropagation of (mg l^{-1})

Stages of micropropagation	IBA (mg l^{-1})	BAP (mg l^{-1})	GA_3 (mg l^{-1})
Introduction into culture	0.1	0.3	0.1
Multiplication	0.01	0.5	–
Rooting	0.2	–	–

The plants were grown in a growth chamber with a controlled temperature of 22 ± 2°C, 16/8 h (day/night) photoperiod and illumination of 2000–3000 lx. The subculturing period was 30 days.

Adaptation of plants to ex vitro environmental conditions

Rooted plants with a stem height of over 3 cm and a well-developed root system were subjected to cold stress for one day at 6–7°C, after that they were planted in a sterile substrate peat-sand (1:1). To prevent fungal pathogens development, they were treated by 0.1% fungicide solution. The adaptation process took place in polyethylene tunnels inside a glass greenhouse at a temperature of 20–22°C and a gradual reduction in air humidity for a month.

Results and Discussion

The analysis of the results of the applied unified sterilization system shows that the lowest percentage of contamina-

Table 1. Origin of raspberry cultivars in the present study

Cultivar	Parents	Selection/Origin
Samodiva	Bulgarian rubin x Shopska alena	Bulgaria
Candidate-cultivar Magdalena	Comet x Lyulin	Bulgaria
Meeker	Willamette x Cuthbert	USA
Willamette	Newburgh x Lloyd George	USA

tion was found in Samodiva cultivar – 8.9%. The infections in the other introduced apical buds of the tested cultivars were respectively 40% for Magdalena, 72% for Meeker and 74.8% for Willamette, indicating the need to further optimization the sterilization conditions according to each genotype.

Impact of genotype on in vitro proliferation

The resulting average number of shoots per single explant is dependent on the genotype and the number of passages. Figure 1 presents the average data for six consecutive passages of micropropagated raspberry cultivars.

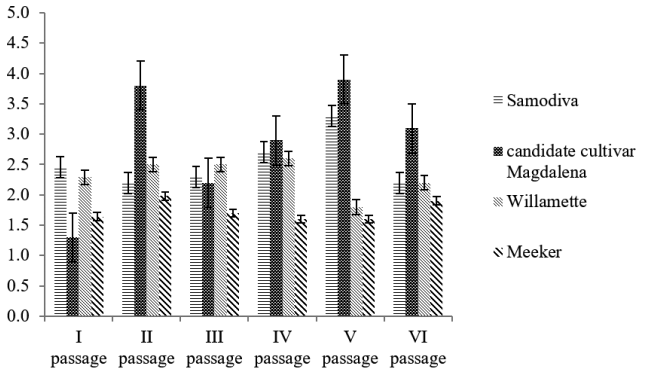

Fig. 1. Average shoot number per explant in different raspberry genotypes (± SE)

The highest multiplication coefficient (3.9) was established in Magdalena, followed by Samodiva (3.3) in the fifth passage and Willamette (2.6) in the fourth passage. There were no statistically proven differences between the passages. A tendency to gradually increase the average number of regenerants in each subsequent passage was found. In the case of Samodiva and Magdalena to the VI passage, and Willamette to V passage, while in Meeker the multiplication coefficient remained similar and the lowest (varying from 1.6 to 1.9). The differences between Magdalena and Meeker cultivars were mathematically proven. The highest (34.5%) average variance in the values was recorded for Magdalena (Table 3).

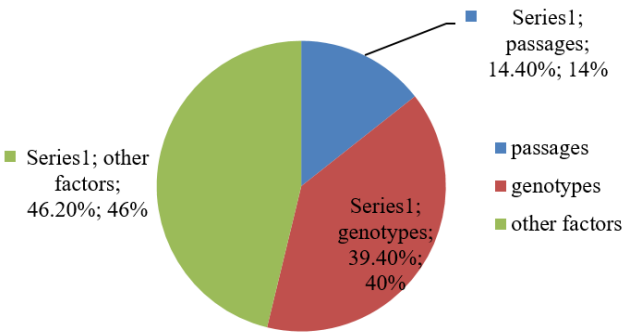

Fig. 2. Percentage impact of the factors on the proliferation coefficient

Figure 2 reflects the impact of the factors on the induction of proliferation coefficient. The highest impact was established for the other factors – 46.20%, followed by the genotype – 39.40% and the lowest impact had the passages.

The highest value of the average length of newly-formed shoots was found in Magdalena on the fourth (3.76 cm) and fifth passage (3.34 cm) (Figure 3). The weakest growth was recorded on the second passage of Willamette cultivar (1.51 cm). There were no statistically proven differences between the passages, but they were established comparing the genotypes, in particular, Willamette with Samodiva and Magdalena, and between Meeker and Magdalena. The lowest variation coefficient was found for Willamette (6.3%) and the highest for Magdalena (20.7%) (Table 4).

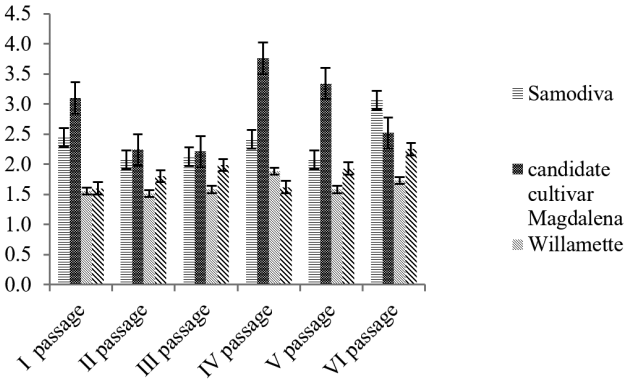

Fig. 3. Average shoots length in raspberry genotypes (±SE)

Table 3. Statistical analysis of multiplication potential in raspberry cultivars for different passages

Genotype	Samodiva	Candidate-cultivar Magdalena	Willamette	Meeker
St Dev	0.4	1.0	0.3	0.2
Amplitude min – max	2.2–3.3	1.3–3.9	1.8–2.6	1.6–2.0
CV	16	34.5	13	11.8
LSD 0.05	1.04			

Table 4. Average length (cm) of the shoots in the tested cultivars by different passages

Genotype	Samodiva	Candidate-cultivar Magdalena	Willamette	Meeker
St Dev	0.4	0.6	0.1	0.2
Amplitude min – max	2.1-3.1	2.2-3.8	1.5-1.9	1.6-2.3
CV	16.7	20.7	6.3	10.5
LSD 0.05	0.71			

The analysis of the data on the average shoot length showed that the impact of the other factors had the highest percentage – 63.50%, followed by the genotype – 27.10% and the number of the passages – 10.60% (Figure 4).

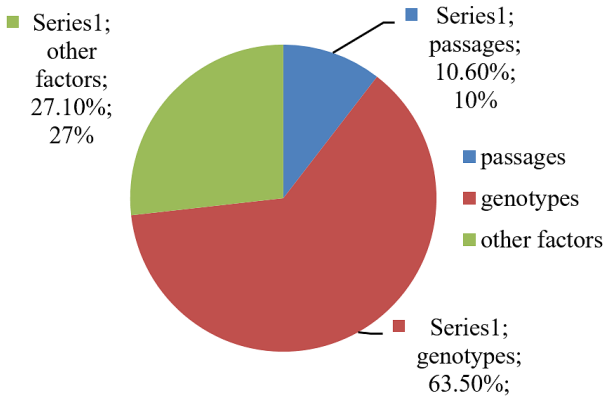

Fig. 4. Impact of the factors on the indicator average shoot length

The results obtained showed that for the raspberry cultivars studied, the regeneration response was genotypically dependent, and the micropropagation ability is in favor of Magdalena cultivar, which had the most significant number of newly-formed shoots.

Regarding the choice of MS basal cultural medium (with high potassium and ammonium ions) our results are in line with the data obtained from Sobczykiewicz (1992). The application of the same combination of growth regulators, although at higher concentrations (0.1 mg l^{-1} IBA, 1-3.0 mg l^{-1} BAP), and the supplement of 50.0 mg l^{-1} ascorbic acid, turned to be the most suitable for the multiplication of raspberries.

Similar results were also observed by Stoevska et al. (1995), who developed an effective system for micropropagation of Bulgarian raspberry cultivars Shopska Alena and Samodiva. The authors recorded proliferation capacity of 6.86 shoots per explant for Shopska Alena and 5.53 respectively for Samodiva, enriching the culture medium with cytokinin (1.0 mg l^{-1} BAP).

In vitro rooting of raspberry plants

Our studies on the optimization of the rooting of raspberries under *in vitro* conditions were performed by testing a cultural medium with reduced mineral composition in half (½MS) and a low auxin concentration (0.2 mg l^{-1} IBA). In the experiment, the root formation of 80.5% was recorded in Samodiva and 26.5% for Magdalena cultivar (Figure 5). Regarding the selection of auxin, it was consistent with the data of Velchev (2004), achieving rooting from 42.50% for Iskra to 84.73% for Vetten cultivar using 1.0 mg l^{-1} IBA. In our previous studies, the impact of different types of auxins, vitamins and organic supplements (mesoinositol and casein hydrolyzate) on the raspberry's rooting capacity was studied (Georgieva, 2006).

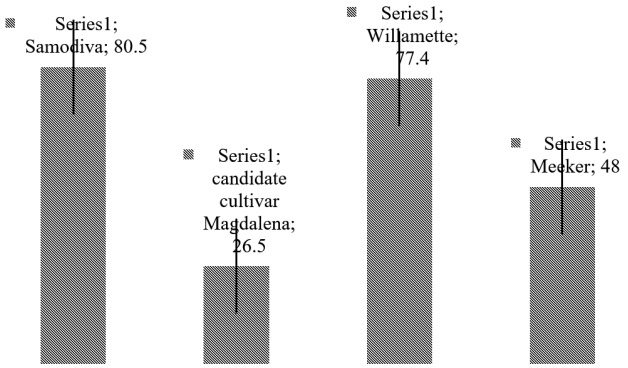

Fig. 5. Effectiveness of rooting in the studied raspberry varieties (± SE)

Concerning the mineral composition of the culture medium, Stoevska et al. (1995) achieved the highest rooting rate on ½ MS, enriched with 0.5 mgl^{-1} IBA, as for Shopska Alena it was 100%, and 93.3% for Samodiva.

Isac and Popescu (2009) studied the rhizogenesis in 26 raspberry cultivar samples, testing different types and combinations of auxins, and found that IBA at concentrations of 0.5, 1 or 2 mg l^{-1} was the best for root induction. In Willamette cultivar, they got 69.9% rhizogenesis, supplementing the culture medium with 2.0 mg l^{-1} IBA and 162 mg l^{-1} PG (phloroglucinol).

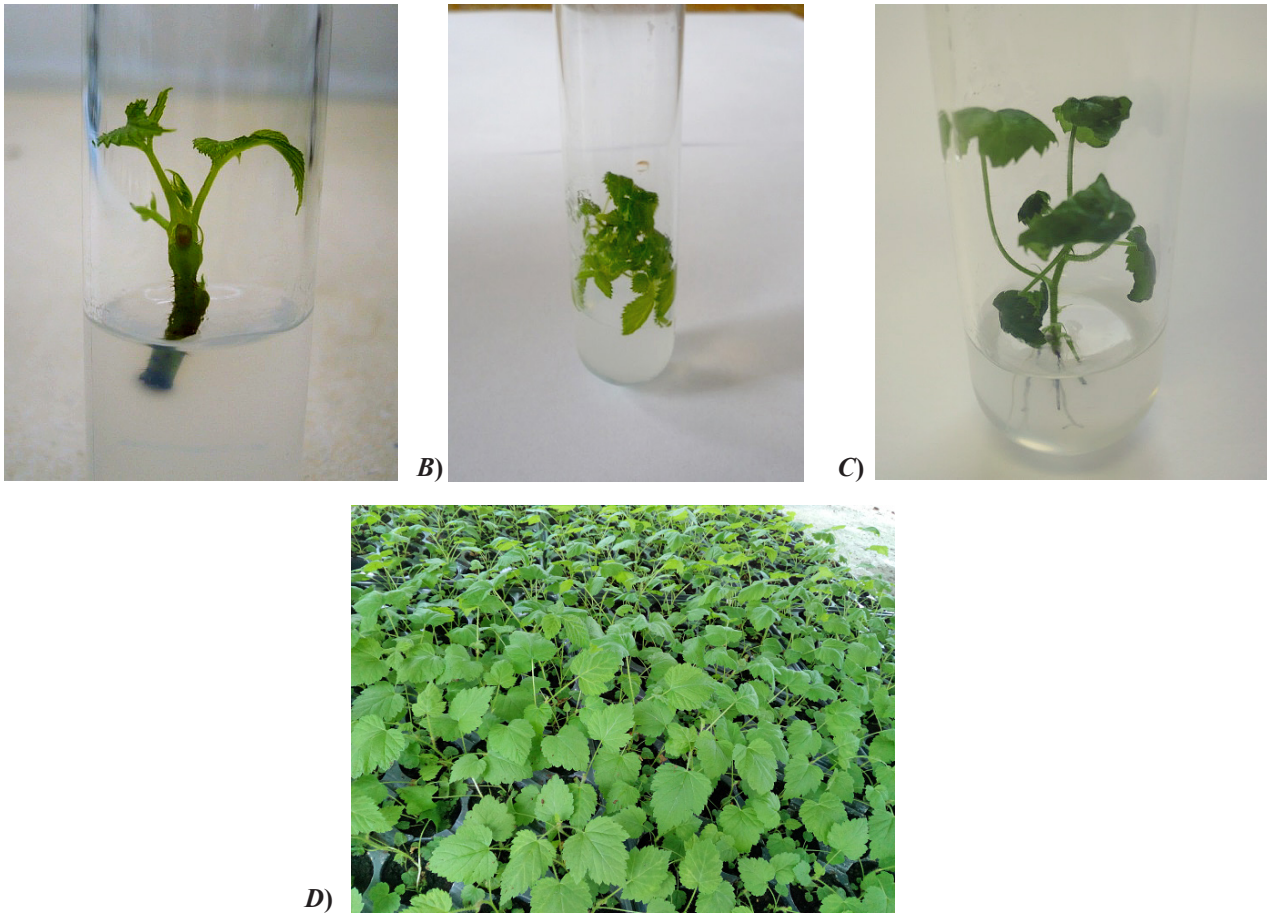

Fig. 6. Stages in raspberry micropropagation: (*A*) Establishment in culture, (*B*) Proliferation, (*C*) Rooting and (*D*) Development of plants following acclimatization

Rušič & Lazić (2004) successfully rooted *in vitro* Willamette by application 1 or 2 mg l^{-1} IBA, 0.1 mg l^{-1} GA_3 and 1 mg l^{-1} activated charcoal and reduced mineral content in the basal MS medium. Administration of the lower level of auxin (1 mg l^{-1} IBA) correlated with better rooting – 49.3% compared to 38.1% with a higher concentration.

These experiments re-confirm that *in vitro* rooting of raspberries is dependent on the type and concentration of auxin, on the presence of endogenous (naturally occurring) auxins in the plant cells, and the biological characteristics of the cultivar. Some aspects of our micropropagation system are related to the use of reduced amounts of growth regulators to eliminate the risk of somaclonal variation and the production of vitrified plants.

Adaptation of in vitro propagated raspberry plants

No differences between genotypes were recorded in the adaptation process. Acclimatization of rooted micro-plants to *ex vitro* environmental conditions was effective and more than 90% of them survived in the applied environmental conditions (Figure 6D). The resulting plant material had vitality and homogeneity.

Conclusions

A simplified system for micropropagation of raspberry cultivars Samodiva, Magdalena candidate-cultivar, Meeker and Willamette was developed. The culture establishment was performed on MS basal medium, supplemented with 0.1 mg l^{-1} IBA, 0.3 mg l^{-1} BAP and 0.1 mg l^{-1} GA3.

The highest values of propagation coefficient 3.9 on the fifth passage and average shoots length – 3.8 cm in the fourth passage of subcultivation were recorded for Magdalena candidate-cultivar on MS medium enriched with 0.01 mg l^{-1} IBA and 0.5 mg l^{-1} BAP.

The raspberry cultivars tested were successfully rooted

on MS medium with a reduced salt concentration in half and supplemented with 0.2 mgl^{-1} IBA. Samodiva demonstrated the higher rooting value of 80.5% than the other studied cultivars.

Adaptation to *ex vitro* conditions was successful as more than 90% of plants survived. The optimized water and nutrient regime of container cultivation lead to the production of material with high vitality and stability, well-developed root system and overground matter.

The use of simplified media for the micropropagation of all raspberry genotypes studied is a prerequisite for commercial application and obtaining pre-basic material for planting in the field and further research.

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