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Propagation of blackberry cultivars in three *in vitro* culture systems and evaluation of genetic uniformity

Înmulţirea unor soiuri de mur în trei sisteme de micropropagare și evaluarea uniformității genetice

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ABSTRACT

The present study aimed to evaluate the influence of three *in vitro* culture systems: solid (SM), semi-solid (SSM), and liquid (temporary immersion system-TIS) on the number and quality of proliferated blackberry shoots, along with the analysis of genetic uniformity among the regenerated shoots. The following blackberry cultivars were tested: 'Čačanska Bestrna', 'Chester Thornless', 'Driscoll's Victoria', 'Karaka Black', 'Loch Ness', and 'Polar'. The culture medium used was Murashige and Skoog (MS), with 0.5 mg/L 6-Benzyladenine (BA). SM was gelled with 5 g/L plant agar; for SSM, wheat starch was used at 50 g/L (w/v), and a Plantform bioreactor was used for TIS. The results revealed that, for all varieties, the highest number of shoots per initial inoculum was obtained in TIS, with the 'Karaka Black' variety presenting a maximum of 93.90 ± 4.01. Considering the length of the proliferated shoots, all tested cultivars showed remarkable results in the SSM culture system gelatinized with 50 g/L wheat starch. The highest shoot length values were observed in 'Čačanska Bestrna', with values of 6.89 ± 0.52 cm, followed by 'Chester' with 5.57 ± 0.28 cm and 'Loch Ness' with 5.36 ± 0.76 cm, respectively. The genetic uniformity of proliferated shoots was evaluated by start codon targeted (SCoT) markers.

Keywords: *Rubus fruticosus* L., agar, Plantform, wheat starch, molecular markers

REZUMAT

Scopul prezentului studiu a fost de a evalua influența a trei sisteme de cultură *in vitro*, solid (SM), semisolid (SSM) și lichid (sistem cu imersie temporară -TIS) asupra numărului şi calității lăstarilor de mur proliferaţi, precum şi analiza uniformității și stabilității genetice între plantele regenerate și planta mamă. Au fost testate următoarele soiuri de mur: 'Čačanska Bestrna', 'Chester Thornless', 'Driscoll's Victoria', 'Loch Ness', 'Polar' si 'Karaka Black'. Mediul de cultură folosit a fost Murashige şi Skoog (MS) suplimentat cu 0,5 mg/L 6-Benziladenină (BA). SM a fost gelificat cu 5 g/L Plant agar, SSM a fost gelificat cu amidon de grâu 50 g/L, iar pentru TIS s-a utilizat bioreactorul Plantform. Rezultatele obţinute au arătat că cel mai mare număr de lăstari/inocul iniţial s-a obţinut în TIS la toate soiurile, cu un maxim de 93.90 ± 4.01 prezentat de soiul 'Karaka Black'. În ceea ce priveşte lungimea lăstarilor proliferați, toate soiurile testate au prezentat rezultate remarcabile la sistemul de cultură SSM gelificat cu 50 g/L amidon de grâu. Cele mai mari valori privind lungimea lăstarilor au fost observate la soiul 'Čačanska Bestrna', urmat de 'Chester' și 'Loch Ness' cu valori de 6.89 ± 0.52 cm, 5.57 ± 0.28 cm și, respectiv, 5.36 ± 0.76 cm. Uniformitatea genetică a lăstarilor proliferați a fost evaluată prin markeri SCoT.

Cuvinte cheie: *Rubus fruticosus* L., agar, Plantform, amidon din grâu, markeri moleculari

INTRODUCTION

Micropropagation allows rapid multiplication of disease-free, uniform, and high-quality planting material, regardless of the season and meteorological conditions. However, *in vitro* techniques are considered expensive, and to reduce costs, *in vitro* protocols should be optimized whenever possible (Abdalla et al., 2022).

In vitro culture of Rubus was first reported in the 1970s, and since then many studies have been published on the *in vitro* culture of blackberry (Ahmed and Abd Elaziem, 2022). An important stage for *in vitro* growth of Rubus species is shoot proliferation. For example, the success of shoot proliferation of blackberry (*Rubus fruticosus* L.) depends on certain factors such as variety, basal culture media, gelling agents, plant growth regulators (PGRs) and also their combination and concentration, as well as the micropropagation systems used (Wu et al., 2009; Fira et al., 2014; Hunková et al., 2016; Hunková et al., 2020; Uma et al., 2021).

Regardless of the culture system applied, which can be solid, semi-solid and liquid culture media-based, the maintenance of the environmental conditions at an optimal level is a very important technical issue for the proliferation and growth of shoots (Loshyna et al., 2022).

Although *in vitro* propagation of blackberry is still based on solid media and the most used gelling agent is agar (Baghdady, 2021; Muñoz-Concha et al., 2021; Dewir et al., 2022; Samaan, 2022; Topçu, 2022; Poothong et al., 2022; Saif and Mohamed, 2023), alternative gelling agents less expensive as compared to the relatively high cost of agar, such as Gelcarin GP-812, Isubgol, guar gum, wheat starch, and corn starch have been successfully tested in the *in vitro* multiplication phase of blackberry (Fira et al., 2009; Clapa et al., 2023). Furthermore, recent reports point out that the temporary immersion system (TIS) in liquid media can be successfully used for the mass propagation of varieties such as 'Tupy' (Ayub et al., 2019), 'Black Diamond', 'Black Pearl', 'Chester', 'Triple Crown' and 'New Berry' (Umarusman et al., 2020). Thus, according to Debnath (2011), the large-scale use of liquid cultures and automation of the immersion system represent a sustainable solution to solve the problem of manual handling of plantlets at the *in vitro* proliferation stage.

An important objective of large-scale production of planting material using *in vitro* culture systems is to obtain true-to-type plants (Hamdeni et al., 2022; Santra and Ghosh, 2023; Sharma et al., 2023). Somaclonal variations can occur during the *in vitro* culture of plants and DNA-based molecular markers such as Start codon targeted (SCoT) markers can be successfully used to confirm, at the molecular level, the uniformity of *in vitro* raised plants (Clapa and Hârța, 2021; Bansal et al., 2022; Koppula et al., 2023).

Thus, the present study aimed to evaluate the proliferation and shoot growth of six *R. fruticosus* varieties ('Čačanska Bestrna', 'Chester Thornless', 'Driscoll's Victoria', 'Karaka Black', 'Loch Ness', 'Polar') using three different micropropagation systems: (a) solid medium gelled with agar, (b) semi-solid medium gelled with wheat starch, and (c) a temporary immersion system (TIS) with liquid medium. Another objective of the present study was to assess the genetic identity between the proliferated shoots and their mother plants from each micropropagation system after twelve successive subcultures, using SCoT molecular markers.

MATERIALS AND METHODS

Plant material and in vitro shoot cultures

The plant material used for all experiments was provided from the 12th successive subculture of six blackberry cultivars, and the duration of each subculture was 60 days. The subcultures were maintained on Murashige and Skoog (MS) (Murashige and Skoog, 1962) culture medium supplemented with 0.5 mg/L 6-benzyladenine (BA) and gelled with 5 g/L plant agar or with 50 g/L wheat starch.

The blackberry cultivars included 'Čačanska Bestrna', 'Chester Thornless', 'Driscoll's Victoria', 'Karaka Black', 'Loch Ness', and 'Polar'. In the present study, three *in vitro* culture systems were used: (1) based on solid culture medium (SM) jellified with agar, (2) with semi-solid

medium (SSM) jellified with wheat starch, and (3) using liquid culture medium through a temporary immersion system in a bioreactor (TIS). The culture media used in all three systems was MS supplemented with 0.5 mg/L BA.

For the SM-based micropropagation system, jellification was performed with Plant agar at 5 g/L (w/v), and for the SSM, wheat starch was used at 50 g/L (w/v). The plants were cultured in 720 mL jars with a diameter of 9 cm and a height of 13.5 cm. In each vessel, 100 ml of culture media was added. (Figure 1). For plants cultivated in TIS, the Plantform bioreactor (Welander et al., 2014) was employed with 400 mL of media per bioreactor (Figure 1). The immersion time was one minute every four hours, and aeration occurred once per hour, lasting 4 minutes.

The liquid culture media and those jellified with agar were sterilized for 20 minutes in the autoclave at 121 °C and 0.11 MPa, while those jellified with starch were sterilized for 30 minutes. Additionally, the pH was adjusted to 5.8. All chemicals used in the *in vitro* culture media were purchased from Duchefa (Biochemie B.V, Netherlands).

In each culture vessel were inoculated five fragments of the shoots (2-3 cm in length). In each bioreactor, ten shoot fragments were inoculated. The *in vitro* cultures were grown at 23 ± 3 °C, 2500 lx/m^2 light intensity (Philips CorePro LEDtube 1200 mm 16W865 CG, 1600lm Cool Daylight) and 16h/8h day/night photoperiod for ten weeks.

Evaluation of genetic uniformity by SCoT molecular markers

In the current study, plant material was represented by leaves from seven proliferated blackberry shoots from each variety and grown in each culture system. The leaves were randomly collected to evaluate the genetic uniformity of the regenerated shoots with their mother (control) plants. Plant material was stored at 4 °C until genetic analysis was performed.

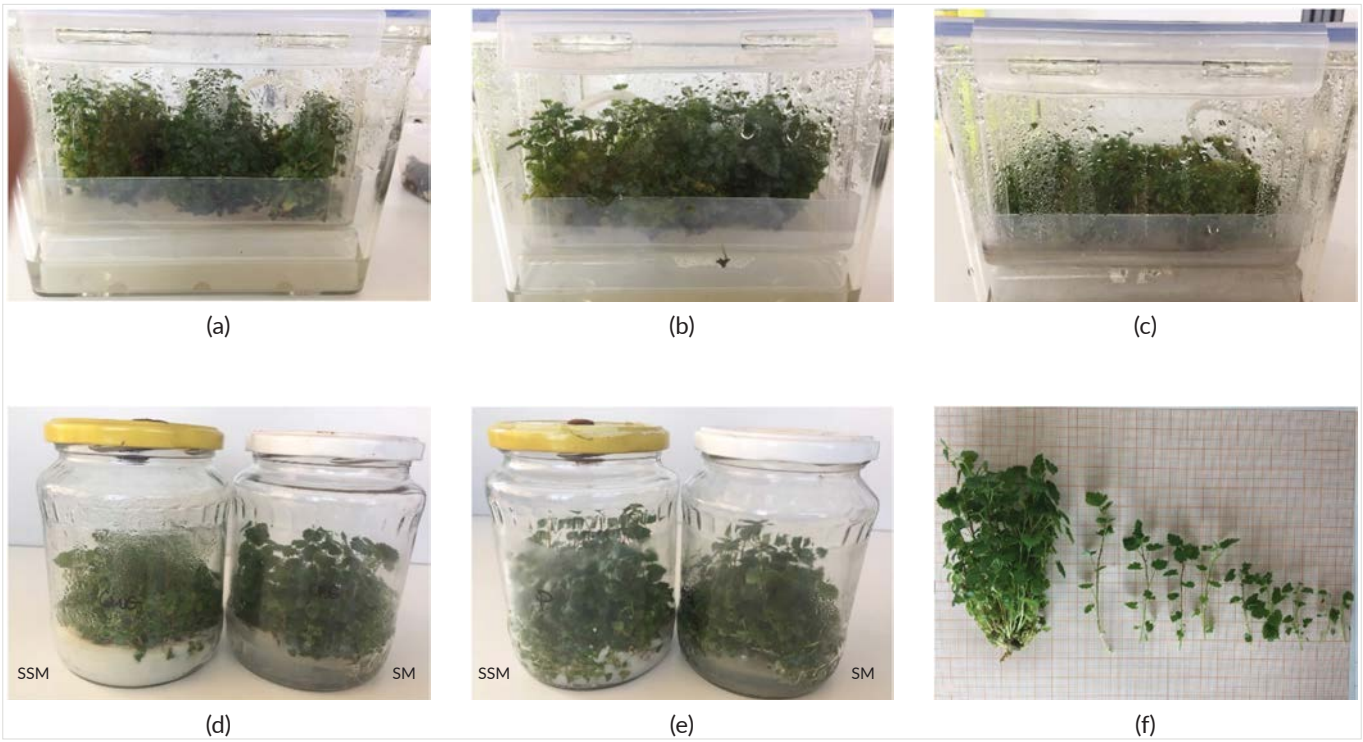

Figure 1. *In vitro* cultures of blackberry, cultivated on MS medium supplemented with 0.5 mg/L BA, in three different culture systems: solid culture medium (SM) jellified with 5 g/L agar, semi-solid medium (SSM) jellified with 50 g/L wheat starch, and liquid culture medium through temporary immersion (TIS) system in Plantform bioreactor: (a) 'Chester Thornless' in TIS; (b) 'Polar' in TIS; (c) 'Karaka Black' in TIS; (d) 'Chester Thornless' in solid (SM) and semi-solid media (SSM), (e) 'Polar' in solid (SM) and semi-solid media (SSM); (f) – 'Loch Ness' – proliferated shoots in semi-solid media.

The extraction of total genomic DNA was performed using a Quick-DNA Plant/Seed Miniprep kit (ZymoResearch, USA) following the protocol described by the supplier company.

PCR analysis: Eight SCoT primers were used in the current study and PCR amplification reactions were performed according to the protocol described by Collard and Mackill (2009) and Hârţa et al. (2023). To assess the reproducibility of the SCoT-PCR amplification results, the experiment was repeated twice for each SCoT primer used. The PCR-amplified products were separated according to the technical conditions described by Hârţa et al. (2023).

Data collection and statistical analysis

Each *in vitro* experiment was repeated three times, more precisely, three jars per repetition and three bioreactors per repetition. After 10 weeks of *in vitro* culturing, the average number of shoots (SN) and shoot lengths (SL) were calculated.

ANOVA was performed followed by Tukey's HSD test ($P \leq 0.05$) to determine the statistically significant differences between the means. Values shown are means ± SE. Agglomerative Hierarchical Clustering (AHC) and heat map were carried out using XLSTAT (New York, NY, USA) and OriginPro 8.0 software (Northampton, MA, USA), respectively.

Captured images of electrophoretic gels were analyzed using TL120 software (Nonlinear Dynamics, Newcastle upon Tyne, UK) to determine the number and molecular weight (bp) range of amplified SCoT bands.

RESULTS

In vitro shoot cultures in solid, semi-solid and liquid media

The three studied culture systems had a distinct influence on the number and length of shoots for all the analyzed cultivars.

In the SM culture system, 'Loch Ness' demonstrated the highest number of shoots (42.27 ± 4.79), significantly surpassing other studied cultivars. Notably, 'Driscoll's Victoria' (19.53 ± 4.86) and 'Chester Thornless' (21.13 ± 3.95) exhibited a reduced number of shoots, as illustrated in Figure 2. Similar to the SM system, the SSM culture system displayed the highest number of shoots per inoculum for the 'Loch Ness' cultivar (52.93 ± 2.51) (Figure 1f). The 'Karaka Black' cultivar also exhibited a substantial number of shoots per inoculum (48.32 ± 2.49), followed by 'Čačanska Bestrna' (47.22 ± 2.13), as depicted in Figure 2.

In the TIS culture system, the 'Karaka Black' cultivar displayed the highest number of shoots per inoculum (93.90 ± 4.01), demonstrating statistically significant differences compared to the other cultivars. Conversely, the 'Polar' cultivar exhibited the lowest number of shoots per inoculum in TIS, with a value of 34.10 ± 1.53 (Figure 2).

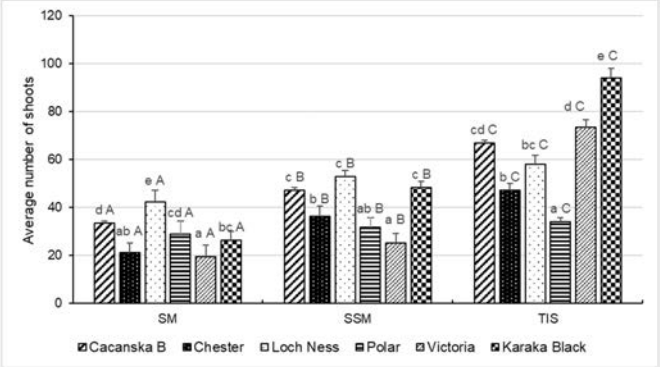

Figure 2. The average number of shoots of the blackberry varieties grown in three different culture systems: solid culture medium (SM) jellified with 5 g/L agar, semi-solid medium (SSM) jellified with 50 g/L wheat starch, and liquid culture medium through temporary immersion (TIS) system in Plantform bioreactor. Different lowercase letters above the bars indicate significant differences between the number of proliferated shoots between cultivars obtained on similar culture systems; different capital letters above the bars represent significant differences between the number of shoots among culture systems of the same cultivars according to Tukey's HSD test ($P \leq 0.05$).

Regarding the length of the shoots obtained, it is evident that most of the analyzed cultivars showed valuable results in the SSM culture system, which was jellified with 50 g/L wheat starch. The highest shoot lengths were observed in the 'Čačanska Bestrna', followed by 'Chester' and 'Loch Ness' with values of 6.89 ± 0.52 cm, 5.57 ± 0.28 cm, and 5.36 ± 0.76 cm (Figure 3).

In the SM culture system, the 'Loch Ness' cultivar demonstrated a noticeable shoot length (5.08 ± 0.86 cm), with statistically significant values compared to the other studied cultivars.

The TIS culture system proved to be suitable for the 'Čačanska Bestrna' cultivar in terms of shoot length, with a mean value of 4.14 ± 0.12 cm for the obtained shoots (Figure 3).

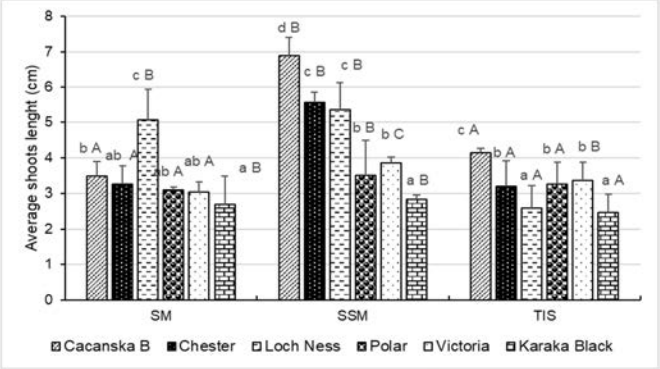

Figure 3. Shoot length of the blackberry varieties grown in three different culture systems: solid culture medium (SM) jellified with 5 g/L agar, semi-solid medium (SSM) jellified with 50 g/L wheat starch, and liquid culture medium through temporary immersion (TIS) system in Plantform bioreactor. Different lowercase letters above the bars indicate significant differences between the means of the shoot length among cultivars obtained on similar culture systems; different capital letters above the bars represent significant differences between the means of the shoot length among culture systems of the same cultivars according to Tukey's HSD test ($P \leq 0.05$).

An observation during the *in vitro* experiments was related to the occurrence of hyperhydricity, also known as vitrification. In the present study, hyperhydricity was notably observed in the shoots propagated in the TIS culture system, with an occurrence rate of 80%, and in the SM culture system, with a rate of 30% across all cultivars. Remarkably, the SSM culture system showed no shoots exhibiting the hyperhydricity phenomenon.

The results of AHC based on the proliferation rate of the six analyzed cultivars obtained on three different culture systems are presented in Figure 4. Accordingly, the cultivars obtained on the SM system (Figure 4a) were classified into three clusters, more exactly class I including 'Čačanska Bestrna', 'Chester Thornless', 'Driscoll's Victoria', and 'Polar', class II with 'Loch Ness' and class III containing 'Karaka Black'.

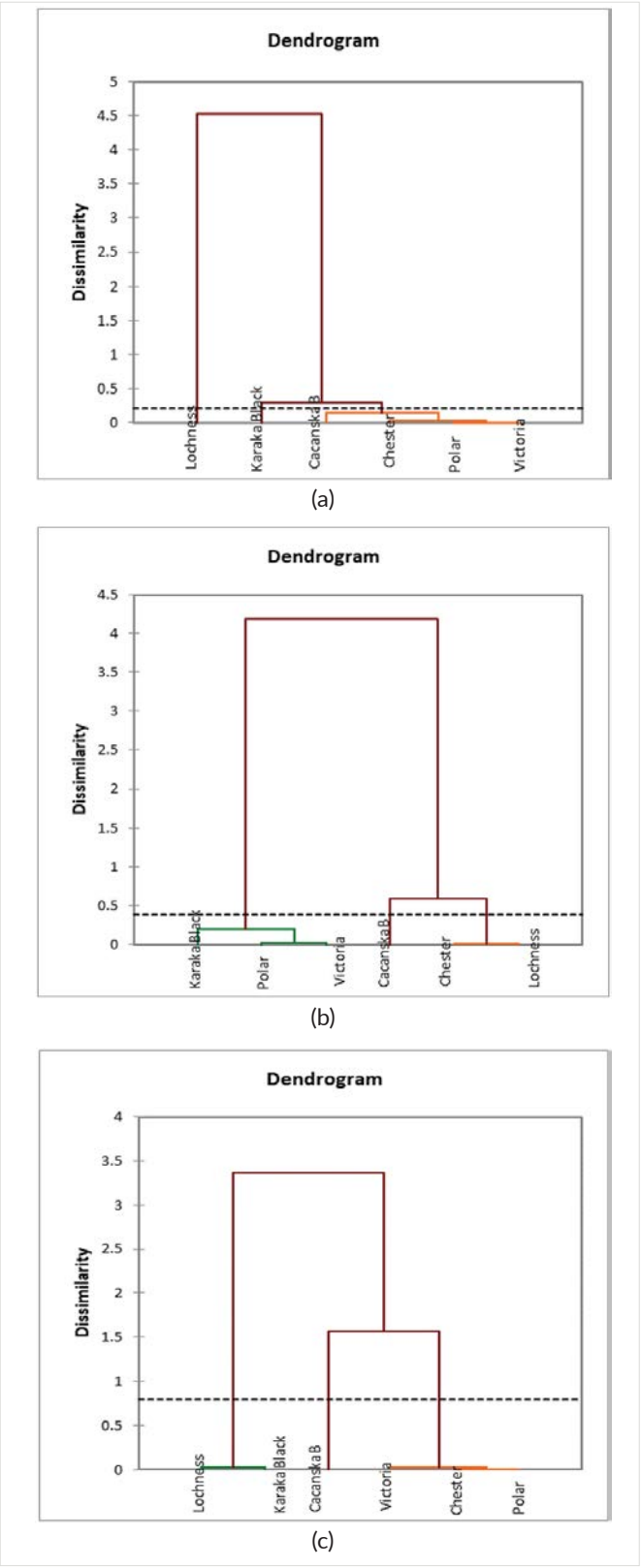

Figure 4. Agglomerative Hierarchical Clustering (AHC) according to the number of shoots/inoculum of the six blackberry cultivars micropropagated in three different culture systems: (a) – solid media (gelled with 5 g/L Plantagar), (b) semi-solid media (gelled with 50 g/Lwheat starch) si (c) liquid media (TIS in PLantform bioreactor)

The cultivars obtained on the SSM system were grouped into three classes as shown in Figure 4b, class 1 contained 'Karaka Black', class two contained 'Chester Thornless' si 'Loch Ness' and class three contained 'Polar', 'Driscoll's Victoria' si 'Karaka Black 'cultivars. The dendrogram that resulted from the AHC of the shoots obtained in the TIS culture system showed three classes; class one contained 'Čačanska Be-strna', class two was composed of 'Chester Thornless', 'Polar' si 'Driscoll's Victoria' and class three encompassed 'Loch Ness' si 'Karaka Black', as shown in figure 4c.

The results of AHC based on the shoot length of the six cultivars on three different culture systems are presented in Figure 5. The blackberry cultivars obtained on the SM culture system were grouped into four classes as illustrated in Figure 5a, class I was composed of 'Čačanska Bestrna', class II contained 'Chester Thornless' and 'Driscoll's Victoria', class III contained 'Loch Ness', and class IV were grouped of 'Polar' și 'Karaka Black'. Both SSM and TIS culture systems generated three classes as shown in Figure 5b and 5c respectively.

A heat map was applied to visualize the variation in the number of shoots/inoculum and shoot length of the six blackberry cultivars in the three *in vitro* culture systems (Figure 6). The growth parameters in SM, SSM, and TIS were clustered according to the Pearson correlation coefficient. The rows denote blackberry cultivars and the columns denote the growth parameters in the three *in vitro* culture systems. Based on the color intensity, a red box shows that the growth parameters were lower than the average level, and a blue box indicates that growth parameters were higher than the average level.

Genetic fidelity evaluation using SCoT molecular markers

Of the eight SCoT primers used for the initial analysis of genetic identity between mother plants and the proliferated shoots/ each variety and culture system, only six SCoT primers generated clear and reproducible DNA-PCR amplified bands.

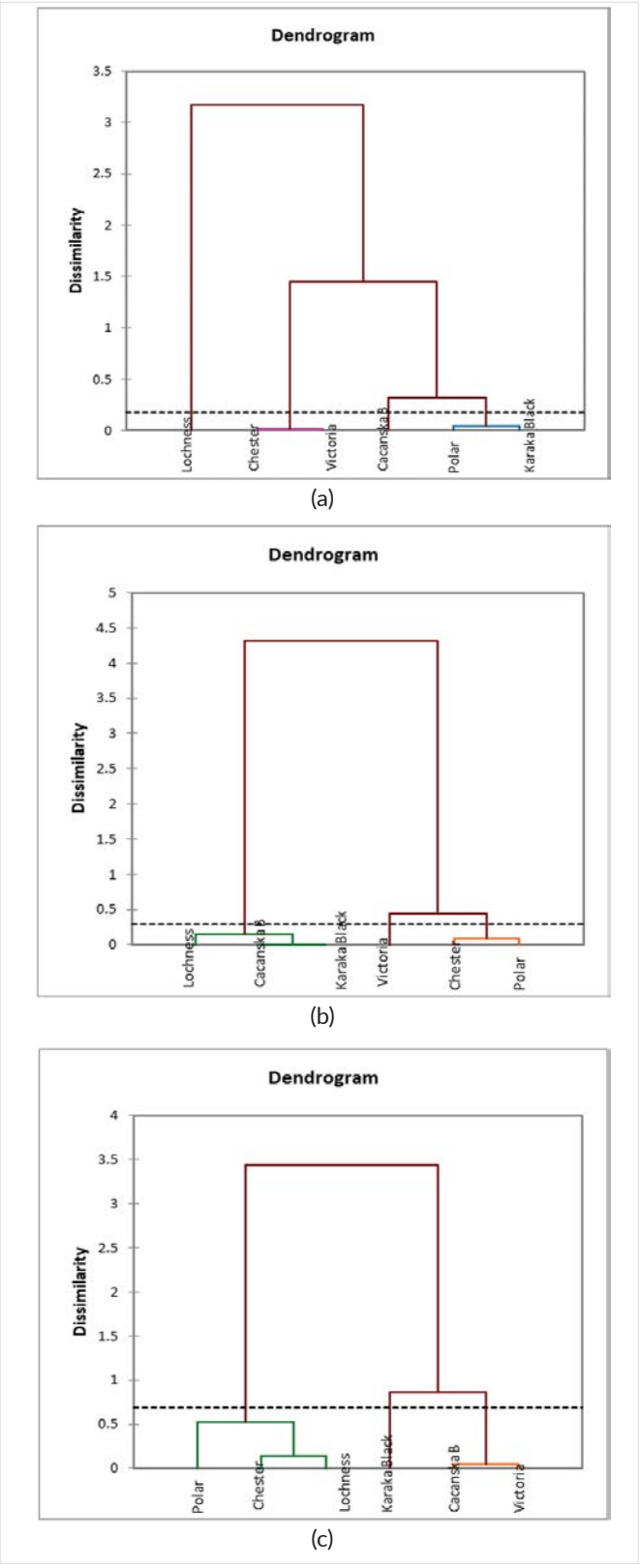

Figure 5. Agglomerative Hierarchical Clustering (AHC) according to the shoots length of the six blackberry cultivars micropropagated in three different culture systems: (a) – solid media (gelled with 5 g/L Plantagar), (b) semi-solid media (gelled with 50 g/Lwheat starch) si (c) lichid media (TIS in Plantform bioreactor)

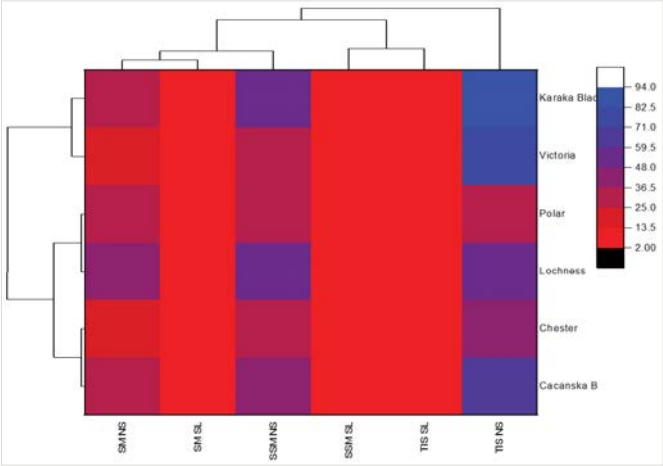

Figure 6. Hierarchical clustering and heatmap visualization of the blackberry cultivars based on the growth parameters in solid (SM), semi-solid (SSM), and liquid medium (TIS). The dendrogram of the columns resulted from the correlation between the growth indicators; the rows' dendrogram shows the correlation between the six blackberry cultivars (SM NS - number of shoots in solid media, SSM NS - number of shoots in semi-solid media, TIS NS - number of shoots in liquid media, SM SL - shoots length in solid media, SSM PR - shoots length in semi - solid media, TIS PR- shoots length in lichid media)

The number and size range of total PCR amplified bands using SCoT primers in the analyzed *R. fruticosus* L. cultivars are presented in Table 1.

The amplified PCR bands ranged in size from 220 bp (SCoT-9; SCoT-14) to 1900 bp (SCoT-9). It should be noted that there were no variations between the numbers of PCR amplified bands recorded for each variety grown *in vitro* in the three culture systems used in this study (Table 1). The number of monomorphic amplified SCoT bands varied between 6 and 16. A total of 6 bands were observed for SCoT-15 in samples 'Čačanska Bestrna' and 16 bands for SCoT-9 in samples 'Karaka Black' and 'Polar' (Table 1).

The highest number of total amplified SCoT bands was detected in 'Polar' (72), and the lowest number of bands was recorded in 'Loch Ness' (58) (Table 1).

The current study analyzed the level of polymorphism between the *in vitro* proliferated shoots and their mother plants and no polymorphic DNA bands were detected. For example, Figure 7 shows the electrophoretic profile generated with primer SCoT-9 in the SSM culture system. As can be seen in Figure 7, there is no variation in the number of PCR amplified bands between mother plants and *in vitro* proliferated shoots of each cultivar studied.

Table 1. The number and size range of amplified SCoT bands detected in the analyzed cultivars of *R. fruticosus* L. using three micropropagation systems

Primer name	Size range of bands (bp)	No. of scorable monomorphic bands																	
		Čačanska Bestrna			Chester Thornless			Loch Ness			Polar			Driscoll's Victoria			Karaka Black		
		SM	SSM	TIS	SM	SSM	TIS	SM	SSM	TIS	SM	SSM	TIS	SM	SSM	TIS	SM	SSM	TIS
SCoT-9	220-1900	15	15	15	14	14	14	10	10	10	16	16	16	9	9	9	16	16	16
SCoT-12	250-1700	10	10	10	9	9	9	8	8	8	14	14	14	10	10	10	9	9	9
SCoT-13	250-1800	10	10	10	12	12	12	11	11	11	8	8	8	9	9	9	8	8	8
SCoT-14	220-1500	11	11	11	10	10	10	9	9	9	9	9	9	10	10	10	10	10	10
SCoT-15	250-1500	6	6	6	10	10	10	9	9	9	11	11	11	14	14	14	11	11	11
SCoT-19	250-1700	11	11	11	14	14	14	10	10	10	14	14	14	9	9	9	9	9	9
Total no. of bands/ cultivar		63	63	63	69	69	69	58	58	58	72	72	72	61	61	61	63	63	63

SM - solid culture medium jellified with 5 g/L plant agar; SSM - semi-solid medium jellified with 50 g/L wheat starch; TIS - liquid culture medium through temporary immersion system in the Plantform bioreactor

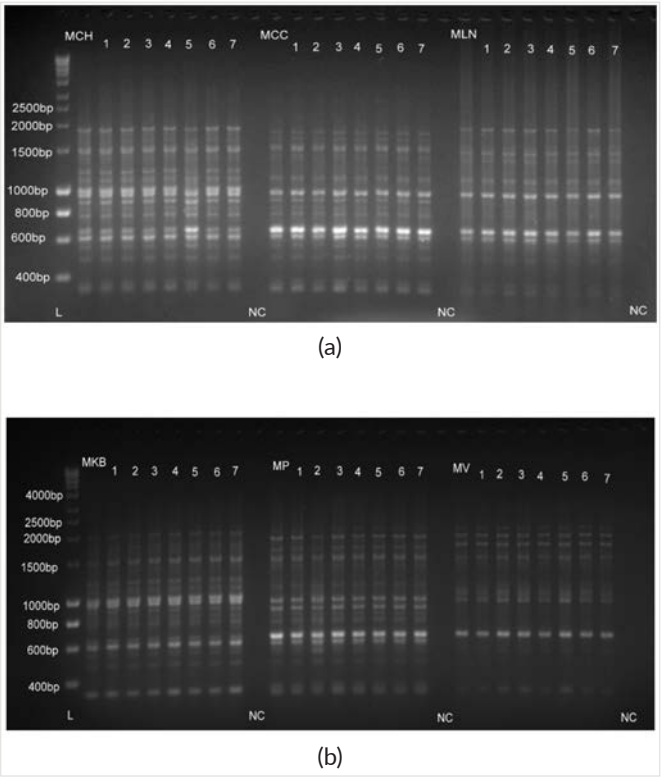

Figure 7. Evaluation of the genetic fidelity between the six *R. fruticosus* L. *in vitro* proliferated shoots, and their mother plants based on SCoT markers. DNA fingerprinting profiles of the mother plants (MCH – Chester, MCC – Čačanska, MLN- Loch Ness (a); MKB - Karaka Black, MP- Polar, MV - Driscoll's Victoria (b) and *in vitro* proliferated shoots in semi-solid medium (1-7) generated by primer SCoT-9; L – molecular marker 1 kb Ladder (Fermentas, Leon-Rot, Germany); NC- sample controls without DNA

DISCUSSION

Previous studies conducted on the micropropagation of different plant species compared the growth parameters of the *in vitro* plants on two types of culture systems, more exactly agar-gelled solid media and liquid media (in different types of bioreactors) (Mehrotra et al., 2007; Georgieva et al., 2016; Aguilar et al., 2019; Umarusman, 2020). To the best of our knowledge, the present study presents the first results on *R. fruticosus* varieties cultivated on three different culture systems, namely solid (gelled with agar), semisolid (gelled with starch), and liquid (in bioreactor) media.

In the current study, the type of culture system had a significant effect on the number of shoots/explant and on the shoot length of the *in vitro* studied blackberry cultivars, as presented in Figures 2 and 3. The highest number of shoots/explant was obtained in the TIS culture system followed by SSM and SM culture systems in all blackberry cultivars. In this regard, the maximal number of shoots/explant was observed in cultivar 'Karaka Black' with a value of 93.90 ± 4.01, which was 1.9 times higher than the same cultivar obtained in the SSM culture system and 3.5 times higher than in SM. The top-most differences in the number of shoots/explant were observed in cultivar 'Driscoll's Victoria', which showed 3.8 times increased values in comparison with the SM culture system and 2.9 times higher values compared to SSM, thus, presenting an evident effect of the culture system on the tested cultivar. Similar results were observed in a study published by Umarusman et al. (2020) regarding 'Black Diamond', 'Black Pearl', 'Chester', 'New Berry' and 'Triple Crown' varieties on two culture systems (TIS and SM) using MS culture media supplemented with 1 mg/L BA. The results of the research showed an increased proliferation rate on TIS, more exactly a two-fold increase was observed compared with the culture systems solidified with agar. The positive effect of TIS was reported previously on the number of shoots of several other species as well. The number of shoots of *Mentha x piperita* produced in Liquid Lab Rocker® (LLR) vessels containing liquid medium (103.4) was significantly higher than those produced on medium jellified with agar (40.7) (Vaidya et al., 2019). An earlier study conducted on Musa spp. reported a higher efficiency of the temporary immersion system (RITA) for rapid propagation, due to the higher number of shoots produced within a relatively short period in comparison with other conventional methods (Jekayinoluwa et al., 2019).

The raspberry, cv. 'Polka' and strawberry, cv. 'Tudla' were propagated in two culture systems (solid media and liquid in TIS bioreactor, RITA® type). The proliferation rate of strawberry plants obtained on a solid medium for 4 weeks presented a value of 1.9 while in liquid media the proliferation rate presented a value of 4.1. For raspberry, the proliferation rate values were 3.2 in SM and 6.7 in TIS (Georgieva et al., 2016). The higher efficiency of TIS might be attributed to the advantage linked to a better affinity of the plant with the culture media, thus assuring

an easier absorption of the nutrients (Jekayinoluwa et al., 2019).

In terms of shoot length, the general tendency was to increase the SSM culture system, which presented the most eloquent results (Figure 3). The cultivar 'Čačanska Bestrna' presented the highest values for shoot lengths in culture media jellified with 50 g/L wheat starch, more exactly 6.89 ± 0.52 cm, which was near twice higher than the values obtained on the SM culture system. A remarkable behavior was observed in 'Loch Ness' cultivar on the tested culture systems (Figure 3). The shoot length of 'Loch Ness' presented the highest values in SSM (5.36 ± 0.76 cm) and the SM culture system (5.08 ± 0.86 cm), while a significant decrease in the shoot length was observed in TIS (2.60 ± 0.61 cm).

The results of previous studies conducted on blackberry cultivars multiplied *in vitro* showed significant differences between the shoot length obtained on solid media and TIS (Umarusman et al., 2020). The cultivars 'Chester', 'Triple Crown' and 'New Berry' presented higher values of the length of the shoot in TIS (Plantofm bioreactor) compared to a solid medium. The same study showed no significant differences in the shoot length between cultivars 'Black Diamond' and 'Black Pearl'. Other plant species cultivated on solid and liquid media showed different tendencies compared to blackberries concerning shoot length. In most of the cases, the *in vitro* obtained plantlets presented higher values for shoot length in TIS compared to solid media. A comparative study of the *in vitro* cultured *Dianthus caryophyllus* cv. 'Tessino Cherry' in solid medium (7 g/L agar) and in a liquid medium, by using a Temporary Immersion Bioreactor (TIB), showed a positive result for the liquid medium through TIB. Precisely the average number of new shoots in solid media was 5.7, with a mean length value of 2.74 cm, while in TIB the average number of new shoots was 14.33 with a mean length value of 4.66 cm (Ahmadian et al,. 2017). The results obtained by Bošnjak et al. (2021) on *R. idaeus* L. cv. HimboTop showed no significant differences between the length of the shoots obtained in the SETIS bioreactor and those obtained in media solidified with 6.3 g/L agar.

In the present experiment, the results highlighted the SSM culture system as the most efficient for the six tested cultivars. The SSM culture system jellified with 50 g/L wheat starch generated high-quality shoots, with appropriate shoot length, suitable for *ex vitro* acclimation. Culture media MS supplemented with 0.5 mg/L BA and solidified with wheat starch generated a high number of shoots/initial explant and vigorous shoots that were suitable for concomitant acclimation and *ex vitro* rooting for other blackberry varieties (Fira et al., 2009; 2012; 2014). The lower viscosity of the wheat starch-gelled medium caused the inocula to sink into the culture medium as they developed and became heavier (Clapa et al, 2023).

Moreover, the shoots obtained in the SSM culture system presented no hyperhydricity (vitrification) phenomenon. This phenomenon is a morpho-physiological disorder observed in the shoots that could be induced by several physical and chemical factors, and which appears mostly in shoots cultured on liquid media (Aguilar et al., 2019). The shoots obtained in our study on the TIS culture system were not suitable for acclimation because of the reduced shoot length and because of the high proportion of shoots affected by the vitrification phenomenon. Regardless, TIS represents a promising culture system for blackberry cultivars which needs further optimization to eliminate the hyperhydricity phenomenon. The optimization processes can be based on testing different concentrations of cytokines, regulation of the number of inoculums per bioreactor or optimization of immersion time and aeration period.

The Agglomerative Hierarchical Clustering (AHC) of the six blackberry cultivars grown *in vitro* confirms that the number of shoots/explant and the shoot length were influenced by both the cultivar and the applied culture system (Figures 4 and 5). The heat map based on Pearson's correlation coefficient showed that the growth parameters were positively correlated with the blackberry cultivars in solid, semi-solid and liquid media. The heat map correlation, presented in Figure 6, confirms that all the studied cultivars revealed the highest number of

shoots/explants in the TIS culture system. Furthermore, the heat map groups 'Karaka black' and "Driscoll's Victoria' cultivars in a class, being representative for the highest number of shoots/explant for the TIS culture system.

Previous research results have shown that assessing the genetic identity between mother plants and their *in vitro* regenerated plants is an important goal in the large-scale production of planting material through tissue culture strategies (Borsai et al., 2020). Although tissue culture is an efficient method of clonal propagation, however, the vitroplants often have several somaclonal variations (Larkin and Scowcroft 1981). These somaclonal variations are mainly caused by newly generated mutations arising from the protocols used in tissue culture as well as due to microenvironmental conditions (Krishna et al., 2016). For example, both culture initiation and subsequent subculture expose explants to oxidative stress (Krishna et al. 2008), which may result in mutations (Cassells and Curry, 2001). In this particular context, DNA-based molecular marker techniques are considered valuable tools for verifying the genetic fidelity of *in vitro* propagated plants compared to their mother plants and for confirming their uniformity for commercial purposes (Clapa et al., 2023).

In the present study, SCoT markers were used to confirm the genetic fidelity of the *in vitro*-grown shoots using three established culture systems (SM, SSM, TIS) after 12 repetitive subcultures. The results are in agreement with those obtained in our previous study showing that SCoT molecular markers can be successfully used to test the uniformity of *Rubus fruticosus* L. plant material with their mother plants (Clapa et al., 2023).

In the current study, three different *in vitro* culture systems were used and the genetic uniformity of regenerated shoots was confirmed. Compared to RAPD or ISSR markers that can amplify DNA fragments from non-coding regions of the plant genome, the SCoT markers are linked with functional genes and their corresponding traits and are considered more efficient for genetic identity studies (Thakur et al., 2021).

CONCLUSIONS

The present study concluded that the most suitable culture system for the six blackberry cultivars ('Čačanska Bestrna', 'Chester Thornless', 'Driscoll's Victoria', 'Karaka Black', 'Loch Ness', and 'Polar') was semi-solid medium. More specifically, MS culture medium supplemented with 5 mg/L BA and gelled with 50 g/L wheat starch gave the most favorable results among the three culture systems, which included solid medium gelled with agar, semisolid medium gelled with wheat starch and liquid medium in the Plantform bioreactor. The genetic uniformity of the proliferated shoots with their mother plants was confirmed by Start codon targeted molecular markers for all *in vitro* culture systems used, revealing the effectiveness of these DNA-based molecular markers for analyzed *R. fruticosus* cultivars.

REFERENCES

Abdalla, N., El-Ramady, H., Seliem, M. K., El-Mahrouk, M. E., Taha, N., Bayoumi, Y., Shalaby, T.A., Dobránszki, J. (2022) An academic and technical overview on plant micropropagation challenges. Horticulturae, 8 (8), 677. DOI: https://doi.org/10.3390/horticulturae8080677

Aguilar, M. E., Garita, K., Kim, Y. W., Kim, J. A., Moon, H. K. (2019) Simple protocol for the micropropagation of teak (*Tectona grandis* linn.) in semi-solid and liquid media in RITA® bioreactors and *ex vitro* rooting. American Journal of Plant Sciences, 10 (7), 1121-1141. DOI: https://doi.org/10.4236/ajps.2019.107081

Ahmadian, M., Babaei, A., Shokri, S., Hessami, S. (2017) Micropropagation of carnation (*Dianthus caryophyllus* L.) in liquid medium by temporary immersion bioreactor in comparison with solid culture. Journal of Genetic Engineering and Biotechnology, 15 (2), 309-315. DOI: https://doi.org/10.1016/j.jgeb.2017.07.005

Ahmed, M. E. A. E., Abd Elaziem, T. M. A. E. (2022) *In vitro* regeneration and improving kaempferol accumulation in blackberry (*Rubus fruticosus* L.) callus and suspension cultures. Egyptian Journal of Chemistry, 65 (12), 369-383. DOI: https://doi.org/10.21608/ejchem.2022.118717.5340

Ayub, R.A., Santos, J.N.D., Zanlorensi, L.A., Silva, D.M.D., Carvalho, T.C.D., Grimaldi, F. (2019) Sucrose concentration and volume of liquid medium on the *in vitro* growth and development of blackberry cv. Tupy in temporary immersion systems. Ciência e Agrotecnologia, 43. DOI: https://doi.org/10.1590/1413-7054201943007219

Baghdady, G. A. (2021) *In Vitro* Propagation of Blackberries (*Rubus* sp) Prime-Ark 45 Cultivar. Annals of Agricultural Science, Moshtohor, 59 (2), 287-294. DOI: https://doi.org/10.21608/assjm.2021.194782

Bansal, Y., Mujib, A., Siddiqui, Z.H., Mamgain, J., Syeed, R., Ejaz, B. (2022) Ploidy Status, Nuclear DNA Content and Start Codon Targeted (SCoT) Genetic Homogeneity Assessment in *Digitalis purpurea* L., Regenerated *In Vitro*. Genes, 13 (12), 2335. DOI: https://doi.org/10.3390/genes13122335

Borsai, O., Hârța, M., Szabo, K., Kelemen, C.D., Andrecan, F.A., Codrea, M.M., Clapa, D. (2020) Evaluation of genetic fidelity of *in vitro*-propagated blackberry plants using RAPD and SRAP molecular markers. Horticultural Science, 47, 21–27. DOI: http://10.17221/20/2019-HORTSCI

Bošnjak, D., Marković, M., Agić, D., Vinković, T., Tkalec Kojić, M., Ravnjak, B., Stanisavljević, A. (2021) The influence of nutrient media modification on the morphological parameters in raspberry (*Rubus idaeus* L.) micropropagation in the liquid and semi-solid media. Poljoprivreda, 27 (1), 22-29. DOI: https://doi.org/10.18047/poljo.27.1.3

Clapa, D., Hârța, M. (2021) Establishment of an Efficient Micropropagation System for *Humulus lupulus* L. cv. Cascade and Confirmation of Genetic Uniformity of the Regenerated Plants through DNA Markers. Agronomy, 11 (11), 2268. DOI: https://doi.org/10.3390/agronomy11112268

Clapa, D., Hârța, M., Szabo, K., Teleky, B. E., Pamfil, D. (2023) The Use of Wheat Starch as Gelling Agent for *In Vitro* Proliferation of Blackberry (*Rubus fruticosus* L.) Cultivars and the Evaluation of Genetic Fidelity after Repeated Subcultures. Horticulturae, 9 (8), 902. DOI: https://doi.org/10.3390/horticulturae9080902

Cassells A.C., Curry R.F. (2001) Oxidative stress and physiological, epigenetic and genetic variability in plant tissue culture: implications for micropropagators and genetic engineers. Plant Cell Tissue Organes Cultures 64, 145–157. DOI: https://doi.org/10.1023/A:1010692104861

Collard, B.C.Y., Mackill, D.J. (2009) Start Codon Targeted (SCoT) Polymorphism: A Simple, Novel DNA Marker Technique for Generating Gene-Targeted Markers in Plants. Plant molecular biology reporter, 27, 86–93. DOI: https://doi.org/10.1007/s11105-008-0060-5

Debnath, S. (2011) Bioreactors and molecular analysis in berry crop micropropagation–A review. Canadian Journal of Plant Science, 91 (1), 147-157. DOI: https://doi.org/10.4141/cjps10131

Dewir, Y. H., Al-Ali, A. M., Rihan, H. Z., Alshahrani, T., Alwahibi, M. S., Almutairi, K. F., Naidoo, Y., Fuller, M. P. (2022) Effects of Artificial Light Spectra and Sucrose on the Leaf Pigments, Growth, and Rooting of Blackberry (*Rubus fruticosus*) Microshoots. Agronomy, 13 (1), 89. DOI: https://doi.org/10.3390/agronomy13010089

Fira, A., Clapa, D., Plopa, C. (2009) Micropropagation of blackberry thornless cultivars. Fruit Growing. Research., 25, 213-221

Fira, A., Clapa, D., Simu, M. (2014) Studies regarding the micropropagation of some blackberry cultivars. Bulletin UASVM Horticulture, 71 (1), 22-37.

Fira, A., Clapa, D., Vescan, L. A. (2012) Direct *ex vitro* rooting and acclimation in blackberry Cultivar 'Loch Ness'. Bulletin UASVM Animal Science and Biotechnologies, 69 (1-2), 247-254.

Georgieva, L., Tsvetkov, I., Georgieva, M., Kondakova, V. (2016) New protocol for *in vitro* propagation of berry plants by TIS bioreactor. Bulgarian Journal of Agricultural Science, 22 (5), 745-751.

Hamdeni, I., Louhaichi, M., Slim, S., Boulila, A., Bettaieb, T. (2022) Incorporation of organic growth additives to enhance *in vitro* tissue culture for producing genetically stable plants. Plants, 11 (22), 3087. DOI: https://doi.org/10.3390/plants11223087

Hârţa, M., Clapa, D., Cornea-Cipcigan, M., Borsai, O., Pop, R., Cordea, M. I. (2023) Multivariate Assessment of Genetic Relationships between Two Streptocarpus Cultivars and Their F1 Progenies Using Morphological Characteristics and SCoT Molecular Markers. Horticulturae, 9 (4), 497. DOI: https://doi.org/10.3390/horticulturae9040497

Hunková, J., Libiakova G., Gajdošová A. (2016) Shoot proliferation ability of selected cultivars of Rubus spp. as influenced by genotype and cytokinin concentration. Journal of Central European Agriculture, 17 (2), 379-390. DOI: https://doi.org/10.5513/JCEA01/17.2.1718

Hunková, J.; Gajdošová, A., Szabóová, M. (2020) Effect of Mesos Components ($MgSO_4$, $CaCl_2$, KH_2PO_4) on *In Vitro* Shoot Growth of Blackberry, Blueberry, and Saskatoon. Plants, 9, 935. DOI: https://doi.org/10.3390/plants9080935

Jekayinoluwa, T., Gueye, B., Bhattacharjee, R., Osibanjo, O., Shah, T., Abberton, M. (2019) Agromorphologic, genetic and methylation profiling of Dioscorea and Musa species multiplied under three micropropagation systems. PloS one, 14, e0216717. DOI: https://doi.org/10.1371/journal.pone.0216717

Koppula, T., Sandhya, D., Rohela, G. K., Kommidi, S., Mohammed, M. (2023) Micropropagation and assessment of genetic homogeneity of regenerants by ISSR and SCoT markers in *Solena amplexicaulis* (Lam.) Gandhi—a threatened medicinal cucurbit. *In Vitro* Cellular & Developmental Biology-Plant, 1-10.

Krishna H, Sairam RK, Singh SK, Patel VB, Sharma RR, Grover M, Nain L, Sachdeva A (2008) Mango explants browning: effect of ontogenic age: mycorrhization and pre-treatments. Scientia Horticulturae, 118, 132–138. DOI: https://doi.org/10.1016/j.scienta.2008.05.040

Krishna, H., Alizadeh, M., Singh, D., Singh, U., Chauhan, N., Eftekhari, M., Sadh, R. K. (2016) Somaclonal variations and their applications in horticultural crops improvement. 3 Biotech, 6, 1-18. DOI: https://doi.10.1007/s13205-016-0389-7

Larkin P, Scowcroft W (1981) Somaclonal variation- a novel source of variability from cell cultures for plant improvement. Theoretical and Applied Genetics, 60, 197–214. DOI: https://doi.org/10.1007/BF02342540

Loshyna, L., Bulko, O., Kuchuk, M. (2022) Adventitious regeneration of blackberry and raspberry shoots and the assessment of the LED-lighting impact. Zemdirbyste-Agriculture, 109, 49-54. DOI: https://doi.org/10.13080/z-a.2022.109.007

Mehrotra, S., Goel, M.K., Kukreja, A.K., Mishra, B.N. (2007) Efficiency of liquid culture systems over conventional micropropagation: A progress towards commercialization. African Journal of Biotechnology, 6 (13), 1484-1492.

Muñoz-Concha, D., Quintero, J., Ercişli, S. (2021) Media and hormones influence in micropropagation success of blackberry cv.'Chester'. Research Journal of Biotechnology,16 (5), 103-108.

Murashige, T., Skoog, F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum, 15 (3), 473-497.

Poothong, S., Kaeothom, N., Chumphukam, O. (2022) Effects of culture medium components and container type on growth and development of blackberry micropropagation. Naresuan Phayao Journal, 15 (2).

Saif, M. I., Mohamed, F. H. (2023) Influence of Plant Growth Regulators and Dark Pretreatment on Shoot Proliferation, Rooting and Acclimatization of Thorny Blackberry. Egyptian Journal of Horticulture, 50 (2), 135-148. DOI: https://doi.org/10.21608/ejoh.2023.181511.1229

Santra, I., Ghosh, B. (2023) Sustainable production of plumbagin via meta-topolin-mediated high frequency regeneration of genetically true-to-type Plumbago zeylanica L. 3 Biotech, 13 (7), 225.

Samaan, M. S. F. (2022) Micropropagation of Blackberry (*Rubus fruticosus*) cv. Karaka Black. Egyptian Journal of Horticulture, 49 (2), 187-198. DOI: https://dx.doi.org/10.21608/ejoh.2022.151882.1205

Sharma, U., Sikdar, A., Igamberdiev, A.U., Debnath, S.C. (2023) Exploring Genetic and Epigenetic Changes in Lingonberry Using Molecular Markers: Implications for Clonal Propagation. Current Issues in Molecular Biology, 45 (8), 6296-6310. DOI: https://doi.org/10.3390/cimb45080397

Thakur, M., Sharma, V., Chauhan, A. (2021) Genetic fidelity assessment of long term *in vitro* shoot cultures and regenerated plants in Japanese plum cvs Santa Rosa and Frontier through RAPD, ISSR and SCoT markers. South African Journal of Botany, 140, 428–433. DOI: https://doi.org/10.1016/j.sajb.2020.11.005

Topçu, H. (2022) Optimal Propagation and Rooting Mediums in Rubus spp. by *in Vitro* Micropropagation. International Journal of Innovative Approaches in Agricultural Research 6 (3), 205-217. DOI: http://doi.org/10.29329/ijiaar.2022.475.4

Vaidya, B.N., Asanakunov, B., Shahin, L., Jernigan, H. L., Joshee, N., Dhekney, S.A. (2019) Improving micropropagation of *Mentha* × *piperita* L. using a liquid culture system. *In Vitro* Cellular & Developmental Biology-Plant, 55, 71-80. DOI: https://doi.org/10.1007/s11627-018-09952-4

Uma, S., Karthic, R., Kalpana, S., Backiyarani, S., Saraswathi, M. S. (2021) A novel temporary immersion bioreactor system for large scale multiplication of banana (Rasthali AAB—Silk). Scientific Reports, 11 (1), 20371. DOI: https://doi.org/10.1038/s41598-021-99923-4

Umarusman, M. A., Şimşek, Ö., Biçen, B., Serçe, S., Kaçar, Y. A. (2020) Farklı Böğürtlen (*Rubus fruticosus* L.) Çeşitlerinin Klasik ve Yeni Nesil Doku Kültürü Teknikleri ile Mikroçoğaltım Olanaklarının Araştırılması. Alatarım, 19 (2), 75-84.

Welander, M., Persson, J., Asp, H., Zhu, L. H. (2014) Evaluation of a new vessel system based on temporary immersion system for micropropagation. Scientia Horticulturae, 179, 227-232. DOI: https://doi.org/10.1016/j.scienta.2014.09.035

Wu, J. H., Miller, S. A., Hall, H. K., Mooney, P. A. (2009) Factors affecting the efficiency of micropropagation from lateral buds and shoot tips of Rubus. Plant Cell, Tissue and Organ Culture (PCTOC), 99, 17-25. DOI: https://doi.org/10.1007/s11240-009-9571-5