Tissue Culture of *Rubus* sp. by Different Methods and Assessment of Genetic Fidelity of Regenerated Plants Using RAPD

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Summary

Different methods employed in plant tissue culture can provide a valuable source of plants for the horticultural industry and novel germplasm for breeding programs, but the genetic fidelity and lack of somaclonal variation of regenerated plants needs to be verified. In this study, the genetic fidelity of blackberry (*Rubus hirtus* Waldst. & Kit.) plants regenerated *in vitro* was assessed with 11 randomly amplified polymorphic DNA (RAPD) markers. Three routes were assessed: callus induction on Yasuda medium with 6-benzyladenine (BA, 8.88 µM), 1-naphthaleneacetic acid (NAA, 10.84 µM) and glycerol (2%, v/v), somatic embryogenesis on Murashige and Skoog (MS) supplemented with 7.57 µM abscisic acid (ABA), and micropropagation from single nodes on MS basal medium containing NAA (0 and 2.86 µM) and BA (0, 4.44, 8.88 and 17.76 µM). MS medium with 2.86 µM NAA and 8.88 or 17,76 µM BA was the most effective medium for axillary shoot multiplication of *R. hirtus* and *Rubus sanctus* Schreb. from nodal segments. A total of 618 fragments were successfully generated by RAPD and the maximum of loci was observed in primer 1204-209 that show 10 and 69 bands. Genetic similarity exceeded 86% when regenerated plants were compared to mother plants. Based on the RAPD data profile, almost true-to-type plants were produced by different methods of plant regeneration (direct shoot regeneration, somatic embryogenesis and organogenesis).

Key words

DNA extraction, genetic stability, plant regeneration, molecular marker

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Received: January 31, 2021 | Accepted: September 21, 2021

Introduction

Blackberries belong to the Rosaceae family, the subfamily Rosoideae and the genus *Rubus* L. They are often grouped with brambles (Hummer, 1996). The available scientific literature contains many studies about the plant tissue culture of different *Rubus* species from economic, health and biotechnology perspectives (Millan-Mendoza and Graham, 1999). The micropropagation of blackberry was reported in several studies (Bobrowski et al., 1996; Erig et al., 2002; Gajdošová et al., 2006; Najaf-Abadi and Hamidoghli, 2009). In these studies, cytokinins and auxins were used as plant growth regulators for *in vitro* proliferation of axillary shoots, followed by the excision and *in vitro* rooting of axillary shoots on culture media with or without auxins. In addition, adventitious shoots were regenerated from leaf or petiole explants (Meng, 2004; Gupta and Mahalaxmi, 2009; Kim and Dai, 2020) while callus was induced from stem internodes and leaves (Sabooni and Shekafandeh, 2018; Fathy et al., 2018). Blackberry stems can be used as explants as nodal segments or thin cell layers (TCLs) (Teixeira da Silva and Dobránszki, 2019). The development of axillary buds can occur via *in vitro* propagation through nodal segments which are not confined to seasonal limitations. This method of propagating plants *in vitro* can produce healthy, true-to-type plants in several species (Shen et al., 1990). A multicellular system that consist one or a few (3-6) layers of differentiated cells from any organ or tissue is often termed a TCL and transversal TCLs, or tTCLs, are frequently used (Teixeira da Silva and Dobránszki, 2015), the genetic stability of the produced plants is affected by several factors. These may include correct signal perception and transduction, an internal genetic machinery and its ability to respond to signals. The physiological state and origin of the tissue or organ influences the responsiveness of TCLs while stress factors, such as prolonged subcultures or osmotic stress, may affect the genetic stability of TCLs, either through apoptosis or gene silencing (Nhut et al., 2003).

Somatic embryogenesis represents a complete model of totipotency and involves the action of a complex signaling network, as well as the reprogramming of highly regulated gene expression patterns (Méndez-Hernández et al., 2019). Indirect organogenesis via callus initiation is possible to induce organs such as roots, shoots and leaves (Ghosh et al., 2018), although the use of some cytokinins such as thidiazuron can induce somaclonal variation (Dewir et al., 2018). It is essential to screen regenerated plants with molecular markers to validate the tissue culture protocol, including from TCLs, to assess their true-to-type nature (Nhut et al., 2003; Kacar et al., 2006; Bose et al., 2017).

Random amplified polymorphic DNA (RAPD) is a convenient, rapid, cost effective, simple and reliable molecular marker to assess the genetic stability of plant tissue cultured material (Kacar et al., 2006; Teixeira da Silva et al., 2007; Jiang, 2013), including in berries such as *Rubus* spp. (Debnath et al., 2012). Using RAPD, Doina et al. (2019) confirmed that regenerated blackberry plants derived from shoot tip culture were genetically similar to mother plants. Using RAPD to genetically profile regenerants of *Eclipa alba* L. transverse TCLs, Singh et al. (2012) detected no differences between mother plants and regenerated plants. Carvalho et al. (2004), on the other hand, detected polymorphism in micropropagated chestnut plants relative to mother plants after using RAPD.

The genetic resources of *Rubus* in Iran consist of eight species and five inter-specific natural hybrids (Khatamsaz, 1992), including blackberry, *Rubus hirtus* Waldst. & Kit. (syn. *R. lanuginosus* Stev. ex Ser), which is very promising for the fruit industry due to its unique pomological features (few thorns, good berry size and a pleasant taste). The aim of this study was to evaluate genetic stability of *in vitro* plants derived from nodal segments and tTCLs and to optimize plant production.

Materials and methods

Plant regeneration

Five *in vitro* plants that were regenerated from tTCLs of *Rubus hirtus* Waldst. & Kit. 'High prickle' via callus or somatic embryogenesis (Sabooni and Shekafandeh, 2017; 2018) were selected. Briefly, tTCLs were soaked in a solution of ascorbic acid and citric acid (60 mg L^{-1} each) before culture on solid Murashige and Skoog (1962; MS) medium containing 2.32 µM kinetin (KIN), 2.69 µM α-naphthaleneacetic acid (NAA) and 8.88 6-benzyladenine (BA) to produce friable and pale green callus. Embryogenic callus was induced on half-strength MS medium containing 60 g L^{-1} sucrose, 9.76 µM KIN and 7.99 µM BA. MS medium fortified with 7.57 µM abscisic acid (ABA) and malt extract (700 mg L^{-1}) or glutamine (400 mg L^{-1}) encouraged the formation and development of somatic embryos. Yasuda (YA) medium (Yasuda et al., 1985) supplemented with 8.88 µM BA, 10.84 µM NAA and glycerol (2%) promoted somatic embryo development. Germination of somatic embryos and growth of normal plantlets occurred on half-strength MS medium containing 4.88 µM BA, 2.02 µM gibberellic acid (GA3) and 0.05 µM NAA. Three plants derived from somatic embryos were selected for molecular analyses, as were two plants derived from indirect organogenesis (callus) after culture of outer dermal parts of explants on YA medium containing 8.88 µM BA, 10.84 µM NAA and 2% (v/v) glycerol.

In addition, one-year-old micropropagated *R. hirtus* (a sparsely thorny plant) and *R. sanctus* Schreb. (a highly thorny plant) plants derived from the culture of nodal segments were used. Mother plants were collected from nature and after identification they were maintained in the Shiraz University greenhouse. The mother plants used for the research were two years old. The nodal segments of about 1.5 cm were harvested, then rinsed under tap water for 30 min, surface disinfected was done under air lamina cabinet with 70% ethanol for 40 s, then with a solution of 10% Clorox (5.25% sodium hypochlorite) and a household detergent (0.2%) for 20 min, then rinsed thoroughly with sterilized double distilled water. Explants were cultured on MS basal medium supplemented with different concentrations of NAA (0 and 2.86 µM) and BA (4.44, 8.88 and 17.76 µM). To prevent the death of the explants, due to the secretion of phenolic compounds, every three days, several mm of the bottom of the explants were removed and then transferred to the same medium. After 45 days, regenerated axillary shoots were transferred to half-strength MS medium containing indole-3-butyric acid (IBA) or NAA (0, 5.37 or 10.74 µM) and BA (2.22 µM) for root induction. After 45 d, the root induction percentage and root length were determined.

In vitro culture conditions

After adjusting media pH to 5.8, 0.8% agar was added to the all media, which were autoclaved at 1.2 kPa pressure and 121 °C for 15 min. All cultures were grown under white fluorescent light (16-h photoperiod, 45 $\mu mol^{-2}\ s^{-1}$) was applied to all cultures for incubation at 25 ± 2.0 °C and 60-70% relative humidity (RH).

Acclimatization and greenhouse growth conditions

Agar was removed from roots by washing under tap water. Regenerated plants were transferred *ex-vitro* to pots with a sterilized substrate of peat: perlite 1:1 (v/v) and kept in a greenhouse (27–30 °C, 85% RH and natural light) for 30 d. Two plants were also selected from this part (direct regeneration).

DNA extraction and PCR (RAPD) amplification

Genomic DNA was extracted three times for each sample according to the cetyltrimethylammonium bromide (CTAB) method of Doyle and Doyle (1987) from the leaves of an *ex-vitro R. hirtus* donor mother plant and seven *in vitro* derived regenerated plants (two from direct organogenesis /adventitious shoot formation) from nodal segments, three from indirect somatic embryogenesis, and two from indirect (via callus) organogenesis (adventitious shoot formation). DNA was pooled and purity and quality was assessed on a 0.8% agarose gel coupled with a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Genomic DNA was then amplified by PCR using RAPD primers (16 were initially tested, but only 11 were usable; Table 1). PCR samples (20 µL) containing 10 mM of primers, 10 ng of template DNA, 1.75 mM $MgCl_2$, 1× reaction buffer, 2 U of Taq DNA polymerase and 0.25 mM dNTPs mix were amplified in a Bio-Rad My Cycler Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) using the following procedure: denaturation for 5 min at 94 °C, 45 cycles of 1 min at 94 °C, annealing for 1 min (32-36.9 °C depending on the primer), 72 °C for 2 min, and finally an extension step at 72 °C for 10 min. RAPD amplification products were run in triplicate on a 2% agarose gel, including 1 $\mu g\ mL^{-1}$ ethidium bromide in 0.5× TBE buffer (50 V, 90 min) and a 100-bp DNA ladder (Gene ruler DNA Ladder mix, SM 0331, Fermentas, Thermo Fisher Scientific, St. Leon-Rot, Germany). A gel documentation system (Thermo Fisher Scientific China Co. Ltd., Shanghai, China) was used to count the number of bands.

Commercial sources

Plant growth regulators, agar and chemicals such as ethidium bromide were purchased from Merck (Darmstadt, Germany).

Statistical analysis

Experiments were in a completely randomized design, and five replications (with a least five explants per replicate) were used in factorial experiments and repeated twice. Data were analyzed by SAS 9.0 (SAS Institute, Cary, NC, USA) using generalized linear models (GLM). Mean values were compared by the least significant difference (LSD) test ($P \leq 0.05$). Gel bands were scored as 0 (absence) or 1 (presence). Similarity coefficients were calculated by UPGMA (Unweighted Pair Group Method with Arithmetic Mean; Sneath and Sokal 1973). A genetic similarity matrix was generated based on Jaccard's coefficient using Sim Qual module in NTSYS-pc 2.02 and to generate a dendrogram (Rohlf 1998). The same program was then operated through the EIGEN and PROJ modules to enable two-dimensional principal component analysis (PCA). Polymorphism information content (PIC) was calculated according to Oliveira et al. (2006) while the probability of identity (PI) was calculated as outlined by Pollefeys and Bousquet (2003).

Table 1. Characterization of RAPD markers used

Code name	Sequence 5' - 3'	Annealing temp. (°C)	Total no. of bands	Total no. of loci	Polymorphic bands	Polymorphism (%)
1204-209	TCACGTCCAC	35.6	69	10	2	20
1204-214	TCGGCGATAG	35	55	7	1	14.28
1204-215	TTCCGAACCC	36.6	48	6	0	0
1204-216	AGCCAGACGA	36.9	76	10	2	20
1204-218	GTGCCTAACC	32	56	8	2	25
1204-220	AATCGGGCTG	34.4	40	6	3	50
1204-221	GGCACTGAGG	35.6	61	9	2	22.22
1204-225	GGTAGCAGTC	36.9	45	6	1	16.66
1204-226	GGTCCTCAGG	36.9	61	8	1	12.5
1204-230	AGAGTCGCCC	32.9	54	7	2	28.57
1204-231	CAGTTCGAGG	36.9	53	8	4	50

Results

Plant regeneration

MS medium with 2.86 µM NAA and 8.88 or 17.76 µM BA was best for *in vitro* shoot induction (Fig. 1) from nodal explants in both *Rubus* species (Table 2), although the number of shoots per explant in *R. sanctus* (8.98) was significantly higher than in *R. hirtus* (3.52).

Table 2. Effect of different concentrations of BA and NAA on shoot induction from *Rubus hirtus* Waldst. & Kit. and *Rubus sanctus* Schreb. nodal explants

Genotype	BA (µM)	NAA (µM)		Mean of genotype
		0	2.86	
R. sanctus	0	1.28 cde	1.40 cde	
	4.44	1.53 dc	1.60 c	
	8.88	3.42 b	8.62 a	3.80 A
	17.76	3.57 b	8.98 a	
R. hirtus	0	0.63 h	0.72 h	
	4.44	0.90 fgh	0.78 gh	
	8.88	1.20 def	3.30 b	1.51 B
	17.76	1.10 efg	3.52 b	
	Mean of NAA	1.70 B	3.61 A	

Means followed by the same letters (capital letters for main effect and small letters for interactions) are not significantly different ($P < 0.05$; LSD multiple range test; n=10)

The shoots of *R. sanctus* rooted in the medium which was used for proliferation, whereas *R. hirtus* did not show any root initiation in the same medium. In relation to *R. hirtus* rooting, the results showed that the presence of BA (2.22 µM) in the culture medium did not promote any root initiation (Table 3).

Table 3. Influence of different concentrations of NAA and IBA on shoot rooting of *Rubus hirtus* Waldst. & Kit

Auxin	(µM)	Total no. of roots/shoot	Auxin	Root length (cm)	Mean (cm)
NAA	0	0.00 d		0.00 d	
	5.37	1.30 c		0.37 c	
	9.84	1.72 b	1.00 B	0.44 c	0.27 B
IBA	0	0.00 d		0.00 d	
	4.92	1.79 b		0.65 b	
	9.84	3.23 a	1.67 A	2.13 a	0.92 A

In each column, means followed by the same letters (capital letters for main effect and small letters for interactions) are not significantly different ($P<0.05$; LSD multiple range test; n=10)

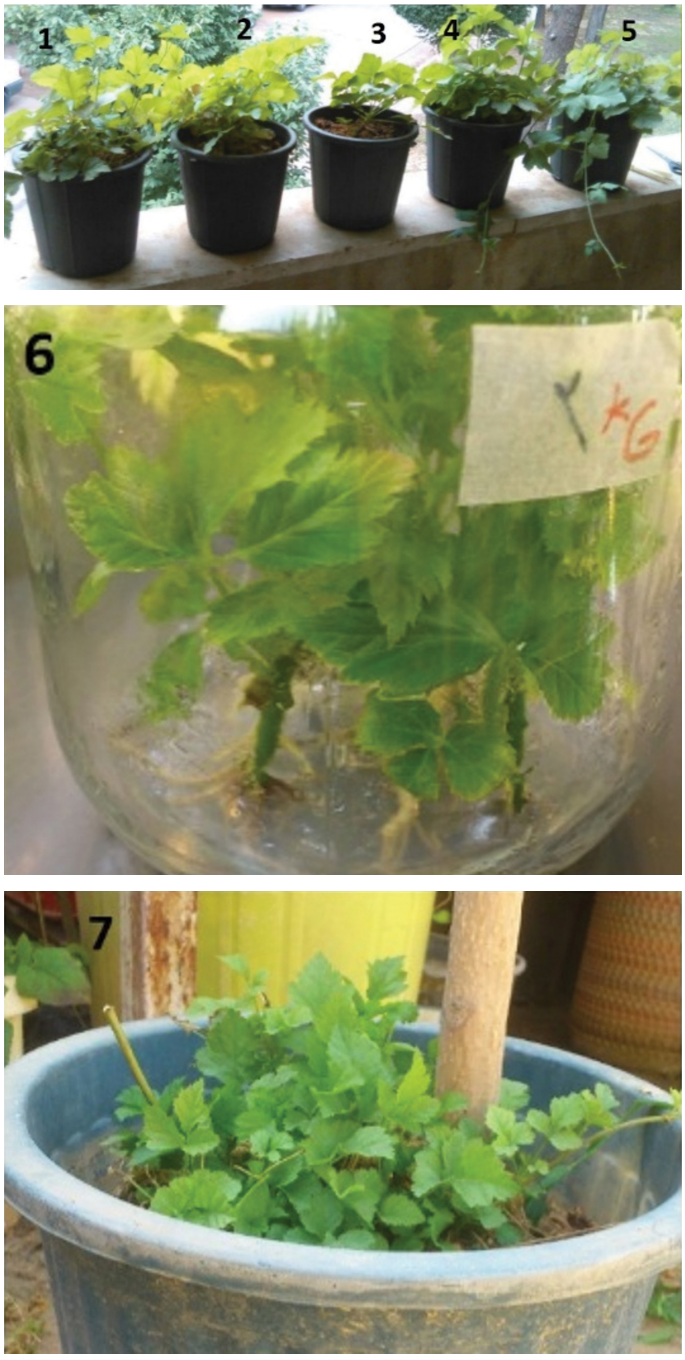

Figure 1. *Rubus hirtus* Waldst. & Kit. plants regenerated *in vitro* via somatic embryogenesis (1, 2 and 3), callus (4 and 5), and nodal segments (6 and 7) and used for RAPD analysis

By adding auxin (NAA or IBA) to the rooting medium, root initiation and elongation occurred. In this regard, IBA was more effective than NAA. The roots and shoots became most abundant when using the medium supplemented with 9.84 µM IBA. The length of roots also significantly increased ($P < 0.05$) on medium containing auxin. The greatest root length was obtained in the medium which contained IBA (9.84 µM) (Table 3).

RAPD profiling and genetic fidelity

RAPD findings for *R. sanctus* were unclear, so they are not presented. Fig. 1 shows surviving *ex vitro* plants. The results using four RAPD primers for *R. hirtus* are shown in Fig. 3. From 11 RAPD primers producing distinct banding, a total of 618 clear bands ranging in size between 200–3000 bp were scored, 78 of which were present in mother plants and in all *in vitro*. Seven bands were polymorphic. Scorable bands ranged between 40 and 76, with an average of 56.18 bands/primer (Table 1, 4). Primer 1204-215 showed the highest polymorphism, and primer 1204-226 showed the maximum loci, PI and PIC (Table 5). Maximum similarity (0.987) was observed between plants 6 and 7, both of which were obtained by nodal micropropagation (Table 6). Lowest similarity (0.8072) was observed between plants 1 and 4, both callus-derived.

The Jaccard's similarity matrix cluster showed that the RAPD has a cophenetic coefficient of 0.91963 based on the Mentel test. The UPGMA-assisted cluster analysis (Fig. 3) showed that the *in vitro* derived plants from all three methods, when pooled, had a higher similarity (92%) to the mother plants than the plant regenerated by somatic embryogenesis (plant 1; 85%). Sub-cluster A contains plants regenerated from callus while micropropagated plants and mother plants formed sub-cluster B (Fig. 3).

The PCA (Fig. 4) showed three groupings: group 1 (plants 2, 3, 5, 6, 7 and 8), group 2 (plant 4), and group 3 (plant 1). The latter, which was derived from somatic embryogenesis, was distinctly distant from the other plants, as was also observed in the cluster analysis (Fig. 3).

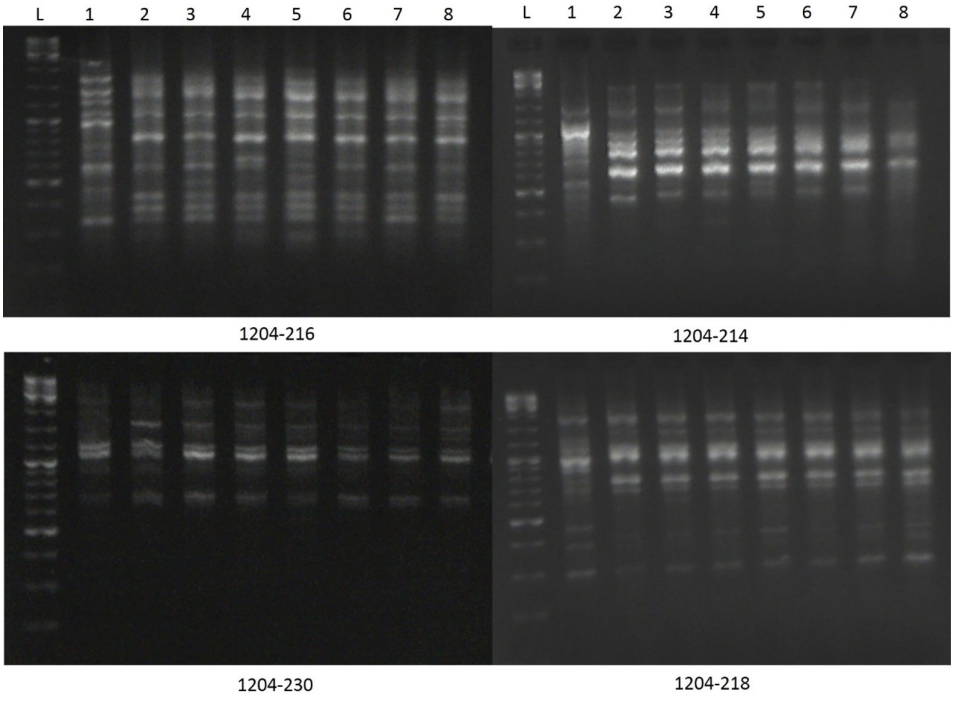

Figure 2. DNA fingerprints based on four RAPD of decamer primers for regenerated *Rubus hirtus* Waldst. & Kit. plants and their corresponding mother plant (codes for lanes in Table 4). Lane L, Gene Ruler DNA Ladder mix SM0331

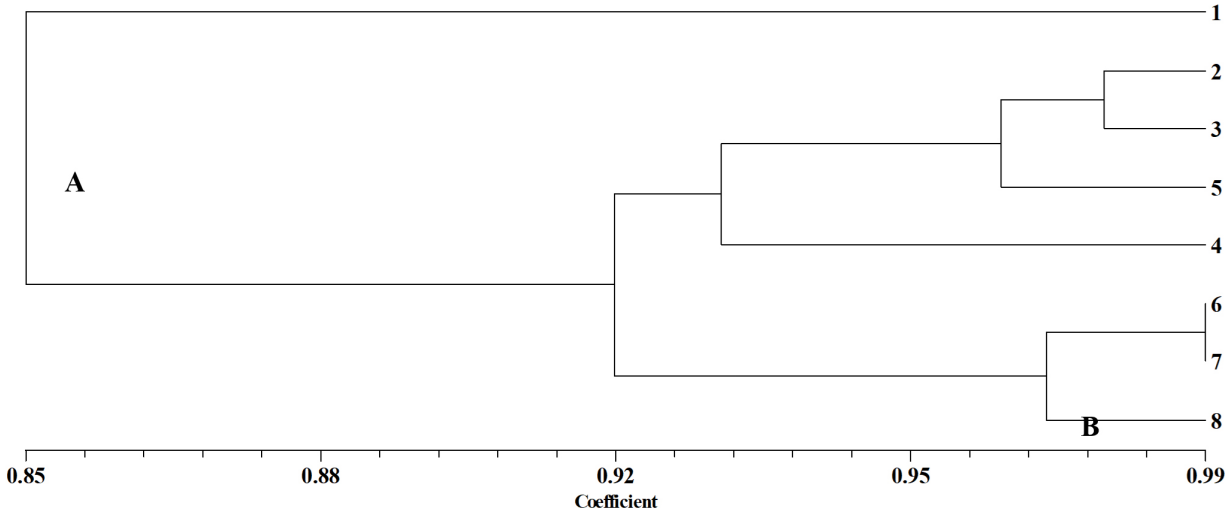

Figure 3. RAPD-derived dendrogram based on Jaccard similarity indices, showing the genetic distance between *in vitro* regenerated *Rubus hirtus* plants (1–7) and donor mother plants (8). Codes for 1-8 explained in Table 4

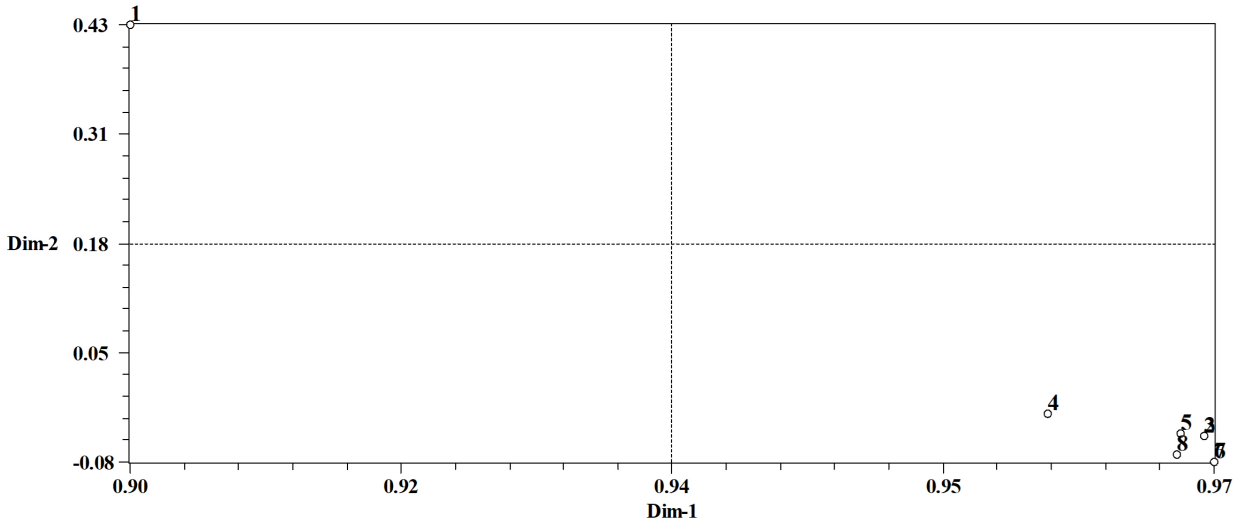

Figure 4. Association among *in vitro* regenerated *Rubus hirtus* Waldst. & Kit. plants (1–7) and donor mother plants (8) as revealed by 2D principal component analysis (PCA) from RAPD data sets. Codes for 1-8 explained in Table 4

Table 4. Total number of bands in seven regenerated *Rubus hirtus* Waldst. & Kit. plants and their corresponding mother plant by 11 RAPD markers

Explant	Method of plant regeneration	Total no. of bands	Plant code number used in Figures
Epidermal strip	Somatic embryogenesis	74	1
		79	2
	Callus	79	3
		76	4
		79	5
Nodal segment	Micropropagation (axillary shoots)	76	6
		77	7
Leaves	Mother plant	78	8

Table 5. Polymorphic information content (PIC), probability identity (PI) and discriminating power (D = 1-PI) of primers used

Primer	PIC	PI	D
1204-209	0.777	0.025	0.975
1204-214	0.799	0.026	0.974
1204-215	0.909	0.034	0.966
1204-216	0.890	0.033	0.967
1204-218	0.912	0.035	0.965
1204-220	0.939	0.042	0.958
1204-221	0.855	0.027	0.973
1204-225	0.763	0.030	0.970
1204-226	0.952	0.045	0.955
1204-230	0.893	0.032	0.968
1204-231	0.894	0.033	0.967

Table 6. Similarity matrixes of the 7 regenerated *Rubus hirtus* Waldst. & Kit. and their corresponding mother plant based on Jaccard's similarity coefficient

	1	2	3	4	5	6	7	8
1	1.0000							
2	0.8430	1.0000						
3	0.8430	0.9750	1.0000					
4	0.8072	0.9375	0.9135	1.0000				
5	0.8658	0.9750	0.9506	0.9375	1.0000			
6	0.8518	0.9375	0.9135	0.9000	0.9375	1.0000		
7	0.8641	0.9500	0.9259	0.8888	0.9500	0.9870	1.0000	
8	0.8536	0.9146	0.8915	0.8780	0.9146	0.9743	0.9620	1.0000

Discussion

The division of plant cells is largely controlled by cytokinins which may favor the direct or indirect initiation of shoots. To promote axillary bud growth and reduce apical dominance, any cytokinin can usually be applied in shoot proliferation medium (George et al., 2007). BA stimulates shoot induction and proliferation in many fruit trees such as guava (Shekafandeh and Khosh-Khui, 2008) and mulberry (Pattnaik et al., 1996). Our findings confirm the results obtained by Najaf-Abadi and Hamidoghli (2009), who reported that the presence of BA (8.88 µM) in MS medium effectively induced shoots in blackberry. However, in contrast to our results, they mentioned that NAA was not an important factor in shoot multiplication, employing GA3 instead of NAA. Fathy et al. (2018) used MS medium with 2.66 µM BA for maximum survival and number of blackberry shoots per explant. Root number and length increased as auxin concentration increased in MS medium. Najaf-Abadi and Hamidoghli (2009) also noted that 9.84 µM IBA was a good stimulator of root induction in blackberry. However, in *Rubus*, Donnelly et al. (1980) noted that MS medium with 2.46-3.44 µM IBA resulted in best root production.

Based on the number of plants that can be induced from a TCL, this is a more cost-efficient method than conventional methods, and can avoid chimerism (Teixeira da Silva and Dobránszki, 2019), which can occur as a result of modifications to DNA methylation, which is related to gene silencing or reactivation of transposable elements (Ma et al. 2018). The recognition of true-to-type germplasm is an important aspect of plant tissue culture, and RAPD is a suitable molecular marker to detect genetic variation in *in vitro* plants (Kacar et al., 2006; Teixeira da Silva et al., 2007; Jiang, 2013), including in *Rubus* spp. (Gajdošová et al. 2006). AbdAlla et al. (2017) used other molecular markers (ISSR and SRAP) to show the true-to-type nature of blackberry plants that had been micropropagated by using 2.22 µM BA. Our findings indicate a high degree of genetic similarity (i.e., limited somaclonal variation) between regenerated plants and their corresponding mother plants, although variation was detected in the somatic embryo route. It is natural to expect various levels of genetic changes (i.e., polymorphism) in plants regenerated *in vitro* via different tissue culture pathways, e.g., direct organogenesis (adventitious shoot induction), callus-induced indirect shoot induction, or somatic embryogenesis. The use of other molecular markers might reveal different levels of genetic variation.

Conclusion

In this study, a suitable regeneration system was established for two *Rubus* species via single node adventitious shoot induction in the presence of BA and NAA. IBA promoted root induction. The genetic (RAPD) profile of plantlets regenerated *in vitro* via three methods (nodal segments, tTCL-induced callus-derived shoot induction (indirect organogenesis), and somatic embryogenesis) were virtually indistinguishable from vegetatively propagated *ex vitro* mother plants. It is important to note that the objective of this paper was not to assess regeneration efficiency or aspects of the tissue culture protocol, including rooting and acclimatization. Future studies should compere original mother plants, explants and regenerants from different regeneration pathways, and using larger sample sizes that used herein, to better appreciate the variation in genetic variation. Such comparisons could be fortified using more than one molecular marker in order to increase the power of detecting genetic variation.

Acknowledgements

The authors thank Mostafa Khoshhal Sarmast (Gorgan University) for guidance and Mohammad Hadi Dehbani (Shiraz University) for technical assistance.

References

AbdAlla M. M., Rashed N. A. K., Abd-Elsalam Yosef M. (2017). Comparative studies between the mother plant and the micropropagated plantlets of blackberry (*Rubus fruticosus* L.). Int. J Curr Res. 9 (12): 62001-62009

Bobrowski V. L., Mello-Farias P. C., Peters J. A. (1996). Micropropagation of blackberries (*Rubus* sp.) cultivars. Curr Agric Res. 2 (1): 17-20. doi: 10.18539/cast.v2i1.146

Carvalho L. C., Goulão L., Oliveira C., Gonçalves J. C. G., Amâncio S. (2004). RAPD assessment for identification of clonal identity and genetic stability of *in vitro* propagated chestnut hybrids. Plant Cell Tissue Organ Cult. 77: 23-27. doi: 10.1023/B:TICU.0000016482.54896.54

Debnath S. C., Vyas P., Goyali J. C., Igamberdiev A. U. (2012). Morphological and molecular analyses in micropropagated berry plants acclimatized under *ex-vitro* condition. Can J Plant Sci. 92 (6): 1065-1073. doi: 10.4141/cjps2011-194.

Dewir Y. H., Nurman S., Naidoo Y., Teixeira da Silva J. A. (2018). Thidiazuron-induced abnormalities in plant tissue cultures. Plant Cell Rep. 37 (11): 1451-1470. doi: 10.1007/s00299-018-2326-1

Doina C., Orsolya B., Monica H., Cristian S. R., Doru P. (2019). Molecular analysis of genetic stability of micropropagated blackberry and blueberry plants using RAPD and SRAP markers. Fruit Grow Res. 35: 79-85. doi: 10.33045/fgr.v35.2019.12

Donnelly D., Stace-Smith R., Mellor F. (1980). *In vitro* culture of three *Rubus* species. Acta Hortic. 112: 69-76. doi: 10.17660/ActaHortic.1980.112.9

Doyle J. J., Doyle J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19: 11-15

Erig A. C., Rossi A. D., Fortes G. D. L. (2002). 6-benzylamino purine and indol butyric acid on the *in vitro* multiplication of blackberry (*Rubus idaeus*), cv. Tupy. Ciência Rural 32 (5): 765-770.

Fathy H. M., El-Leel O. F. A., Amin M. A. (2018). Micropropagation and biomass production of *Rubus fruticosus* L. (blackberry) plant. Middle East J Appl Sci. 8 (4): 1215-1228.

Gajdošová A., Ostrolucká M. G., Libiaková G., Ondrušková E., Šimala D. (2006). Microclonal propagation of *Vaccinium* sp. and *Rubus* sp. and detection of genetic variability in culture *in vitro*. J Fruit Ornam Plant Res. 14 (Suppl. 1): 103-119

George E. F., Hall M. A., De Klerk G. J. (2007). Plant Propagation by Tissue Culture, Volume 1. Netherlands Springer. doi: 10.1007/978-1-4020-5005-3_8

Ghosh A., Igamberdiev A. U., Debnath S. C. (2018). Thidiazuron-induced somatic embryogenesis and changes of antioxidant properties in tissue cultures of half-high blueberry plants. Sci Rep. 8: 16978. doi: 10.1038/s41598-018-35233-6

Gupta S., Mahalaxmi V. (2009). *In vitro* high frequency direct plant regeneration from whole leaves of blackberry. Sci Hortic. 120 (1): 22-26. doi: 10.1016/j.scienta.2008.09.010

Hummer K. E. (1996). *Rubus* diversity. HortScience, 31 (2): 182-183. doi: 10.21273/HORTSCI.31.2.182

Jiang G. L. (2013). Molecular Markers and Marker-Assisted Breeding in Plants. Chapter 3, IntechOpen, Croatia, pp. 45-83. doi: 10.5772/52583

Kacar Y. A., Byrne P. F., Teixeira da Silva J. A. (2006). Molecular markers in plant tissue culture. In: Teixeira da Silva J. A. (Ed.). Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues (Volume II, 1st Edn.), Global Science Books, Ltd., Isleworth, UK, pp. 444-449

Khatamsaz M., Assadi M., Maassoumi A. A. (eds). (1992). Flora of Iran, Rosaceae family. Research institute of forests and rangelands publication, Tehran, Iran (in Persian), pp. 20-35.

Kim C., Dai W. (2020). Plant regeneration of red raspberry (*Rubus idaeus*) cultivars 'Joan J' and 'Polana'. *In Vitro* Cellular and Developmental Biology – Plant 12: 1-8. https://doi.org/10.1007/s11627-019-10051-1

Ma K. F., Zhang Q. X., Cheng T. R., Yan X. L., Pan H. T., Wang J. (2018). Substantial epigenetic variation causing flower color chimerism in the ornamental tree *Prunus mume* revealed by single base resolution methylome detection and transcriptome sequencing. Int J. Mol Sci. 19 (8): 2315. doi: 10.3390/ijms19082315

Méndez-Hernández H. A., Ledezma-Rodríguez M., Avilez-Montalvo R. N., Juárez-Gómez Y. L., Skeete A., Avilez-Montalvo J., de la Peña C., Loyola-Vargas V. M. (2019). Signaling overview of plant somatic embryogenesis. Front Plant Sci. 10: 77. doi: 10.3389/fpls.2019.00077

Meng R., Chen T. H., Finn C. E., Li Y. (2004). Improving *in vitro* plant regeneration from leaf and petiole explants of 'Marion' blackberry. HortScience, 39 (2): 316-320. doi: 10.21273/HORTSCI.39.2.316

Millan-Mendoza B., Graham J. (1999). Organogenesis and micropropagation in red raspberry using forchlorfenuron (CPPU). J Hortic Sci Biotechnol. 74 (2): 219-223. doi: 10.1080/14620316.1999.11511098

Murashige T., Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant. 15 (3): 473-497. doi: 10.1111/j.1399-3054.1962.tb08052.x

Najaf-Abadi A. J., Hamidoghli Y. (2009). Micropropagation of thornless trailing blackberry (*Rubus* sp.) by axillary bud explants. Aust J Crop Sci. 3 (4): 191-194

Nhut D. T., Tran Thanh Van K., Van Le B., Thorpe T. (Eds.) (2003). Thin Cell Layer Culture System, Regeneration and Transformation Applications. Springer Science & Business Media. doi: 10.1007/978-94-017-3522-3

Oliveira E. J., Pádua J. G., Zucchi M. I., Vencovsky R., Vieira M. L. C. (2006). Origin, evolution and genome distribution of microsatellites. Genet Mol Biol. 29 (2): 294-307. doi: 10.1590/S1415-47572006000200018

Pattnaik S., Sahoo Y., Chand P. (1996). Micropropagation of a fruit tree, *Morus australis* Poir. syn. *M. acidosa* Griff. Plant Cell Rep. 15: 841-845. doi: 10.1007/BF00233153

Pollefeys P., Bousquet J. (2003). Molecular genetic diversity of the French-American grapevine hybrids cultivated in North America. Genome 46 (6): 1037-1048. doi: 10.1139/g03-076

Rohlf F. (1998). NTSYS-pc. Numerical taxonomy and multivariate analysis, version 2.02. Exeter Software Setauket, New York, USA

Sabooni N., Shekafandeh A. (2017). Somatic embryogenesis and plant regeneration of blackberry using the thin cell layer technique. Plant Cell, Tissue Organ Cult. 130: 313-321. doi: 10.1007/s11240-017-1225-4

Sabooni N., Shekafandeh A. (2018). Callus induction via thin cell layer culture of two native blackberry genotypes of Iran. Acta Hortic. 1190: 129-134. doi: 10.17660/ActaHortic.2018.1190.22

Shekafandeh A., Khosh-Khui M. (2008). Effects of bud position and culture medium on shoot proliferation from nodal culture of two mature guava cultivars. Asian J Plant Sci. 7 (2): 177-182. doi: 10.3923/ajps.2008.177.182

Shen X. S., Wan J. Z., Luo W. Y. (1990). Propagation *in vitro* of Chinese gooseberry (*Actinidia chinensis*) through the development of axillary buds. Scientia Hortic. 42 (1-2): 45-54. doi: 10.1016/0304-4238(90)90146-6

Singh S. K., Rai M. K., Sahoo L. (2012). An improved and efficient micropropagation of *Eclipta alba* through transverse thin cell layer culture and assessment of clonal fidelity using RAPD analysis. Ind Crops Prod. 37 (1): 328-333.

Sneath P. H. A., Sokal R. R. (1973). Numerical Taxonomy: The Principles and Practice of Numerical Classification. W.H. Freeman and Co., San Francisco, USA, pp. 230-234. doi: 10.2307/2412767.

Teixeira da Silva J. A., Bolibok H., Rakoczy-Trojanowska M. (2007). Molecular markers in micropropagation, tissue culture and *in vitro* plant research. Genes Genomes Genomics, 1 (1): 66-72.

Teixeira da Silva J. A., Dobránszki J. (2015). Plant thin cell layers: update and perspectives. Folia Hortic. 27 (2): 183-190. doi: 10.1515/fhort-2015-0029

Teixeira da Silva J. A., Dobránszki J. (2019). Recent advances and novelties in thin cell layer-based plant biotechnology: a mini-review. BioTechnologia. 100 (1): 89-96. doi: 10.5114/bta.2019.83215

Yasuda T., Fujii Y., Yamaguchi T. (1985). Embryogenic callus induction from *Coffea arabica* leaf explants by benzyladenine. Plant Cell Physiol. 26 (3): 595-597. doi: 10.1093/oxfordjournals.pcp.a076946.

aCS87_25