Evaluation of genetic fidelity of *in vitro*-propagated blackberry plants using RAPD and SRAP molecular markers

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Abstract: The aim of this research was to evaluate the genetic uniformity of blackberry plants (*Rubus fruticosus* L. 'Loch Ness' and 'Chester Thornless') obtained by micropropagation. Genetic uniformity was analysed by using RAPD and SRAP markers. For the *in vitro* multiplication, the slightly modified Murashige and Skoog (1962) basal medium was used, supplemented with 0.5 mg/L 6-Benzyladenine (BA), prepared with tap water and 50 g/L wheat starch as the gelling agent. This culture medium ensured the regeneration of well-developed plantlets, with multiplication rates of more than 42 for both cultivars. The *in vitro* multiplication was carried out in 30 months including 12 subcultures. The plants obtained from the 3rd and 11th subcultures were compared with the mother plants using 64 SRAP primer combinations (eight forward and eight reverse primers) and 20 RAPD primers to check their genetic fidelity. The amplification products were monomorphic in the micropropagated plants and the mother plant. No polymorphism was detected, thus proving the genetic fidelity and uniformity of the micropropagated plants.

Keywords: *Rubus*; 'Chester'; 'Loch Ness'; SRAP; RAPD; tissue culture

Blackberry (*Rubus fruticosus* L.) is a commonly known berry fruit plant being considered a suitable species for vegetative propagation under *in vitro* conditions (Fira et al. 2011, 2014). In general, *in vitro* proliferation of axillary shoots is carried out on a different culture medium, which have, in their composition, different ingredients such as cytokinins or auxins, followed by the excision and *in vitro* rooting of axillary shoots on the culture media with or without auxins (Bobrowski et al. 1996; Erig et al. 2002; Najaf-Abadi et al. 2009; Vujović el al. 2010). The *in vitro* rooted plantlets are then subsequently transferred to *ex vitro* conditions and hardened on various solid substrates (Abdalla, Mostafa 2015).

Through *in vitro* clonal propagation techniques, plants can be propagated rapidly with a high level of efficiency maintaining a large number of elite plant materials characterised by genetic uniformity (Gao et al. 2010; Debnath et al. 2012).

Previous research results show that initiating the *in vitro* culture from different types of initial plant materials such as shoot apical meristems (Atul et al. 2018), mature nodal explants (Ahmad et al. 2013; Moharana et al. 2018) or axillary buds (Goyal

et al. 2012) provided the genetically identical regeneration of the plants compared to the mother plant even after a large number of subcultures. However, true-to-type clonal fidelity is one of the most important requirements in the micropropagation of any plant species. A major problem which occurs is the presence of a somaclonal variation amongst the subclones regenerated from one parental line, as a direct consequence of the *in vitro* culture of the plant cells, tissues or organs (Lakshmanan et al. 2007; Goyal et al. 2012). In this context, molecular techniques become valuable tools to check the genetic fidelity of the *in vitro* propagated plants to confirm their quality for their commercial utility (Bhatia et al. 2011) as they are not influenced by environmental factors and generate highly reliable and sensitive results (Peng et al. 2015). Among the various molecular markers, nuclear DNA-based markers such as RAPD (Random- Amplified Polymorphic DNA) and SRAP (Sequence-Related Amplified Polymorphism) have been successfully employed to assess the genomic stability of the regenerated *in vitro* plants (Lakshmanan et al. 2007; Senapati et al. 2013). The use of these markers is a fast, cost-effective, highly discriminative and reliable technique (Samantaray, Maiti 2010; Bhatia et al. 2011; Robarts, Wolfe 2014). Therefore, the main aim of this study was to evaluate the genetic fidelity of the regenerated *in vitro* blackberry plantlets that resulted from the 9th subculture for the both 'Loch Ness' and 'Chester Thornless' blackberry cultivars. To evaluate the quality of the obtained planting material, molecular markers were used to prove the applicability of this protocol by offering genetic stability and fidelity among the regenerated plantlets and mother plant.

MATERIALS AND METHODS

Plant material. To check the genetic fidelity of the plantlets, leaves were harvested both from the mother plant and from the *in vitro* propagated plantlets (30-months *in vitro* culture) from the two cultivars 'Loch Ness' and 'Chester Thornless'. In total, 10 leaves were randomly selected for harvest from each subculture (9 subcultures, 50 vessels/subculture) before passing the plantlets to the acclimatisation stage. The harvested leaves were dried and stored at 4 °C until the genetic analyses were performed.

The *in vitro* tissue culture initiation was made by using axillary and terminal buds excised from the annual shoots and inoculated on a modified

Murashige and Skoog (1962) medium (MSm) supplemented with 0.5 mg/L 6-Benzyladenine (BA) and gelled with agar (Plant Agar). The culture media were prepared from the Murashige and Skoog (1962) macro- and microelements stock solutions myo-inositol 100 mg/L, thiamine 1 mg/L, pyridoxine 0.5 mg/L nicotinic acid 0.5 mg/L, 30 g/L sugar, 5 g/L Plant Agar. Before sterilising, all the ingredients were added to the medium and the pH was adjusted to 5.8.

In the multiplication stage, a cost-effective modified MS medium (MSm) was prepared using tap water and wheat starch (50 g/L) as the medium solidifier. The culture vessels used in this experiment were 720 mL jars with screw lids, vented with filters made of an autoclavable plastic sponge in which 100 mL of media was distributed. The media gelled with agar were then autoclaved at 121 °C for 20 minutes, while the media gelled with wheat starch have been autoclaved at 121 °C for 30 minutes. Five mini-shoots were introduced into each vessel until they had 2/3–3/4 parts in contact with the culture medium. The cultures were maintained at 24 ± 1 °C under a fluorescent white light (33.6 μmol/m^2·s) during a photoperiod of 16 : 8 h light and dark cycles.

DNA extraction and PCR amplification conditions. The clonal fidelity of the *in vitro*-grown plantlets was tested by using the RAPD and SRAP markers. The DNA was isolated using the CTAB-based method (cetyl trimethylammonium bromide) as published by Lodhi et al. (1994) and improved by Pop et al. (2003) and Bodea et al. (2016). The DNA purity and concentration were determined with a NanoDrop-1000 spectrophotometer. The samples were diluted to 50 ng/μL using sterile distilled water.

To perform the RAPD analysis, 20 decamer primers were used, but only eight yielded scorable amplification patterns for all of the analysed samples that were considered (Table 1).

In the case of the SRAP analysis, 32 primers were initially screened, out of which 12 primer combinations were found reproducible and generated unambiguous amplification profiles for all the samples (Table 2).

For the RAPD analysis, the PCR (polymerase chain reaction) amplification reactions were carried out as described by Williams et al. (1990) following the protocol used by Pop et al. (2011). The reaction mixtures (25 μL total volume) consisted of 5 μL of 50 ng/μL DNA, 9.3 μL distilled H$_2$O for the PCR reactions, 2 μL of PVP (Polyvinylpyrrolidone), 5 μL of a GoTaq Flexi green buffer (Promega Corp., Madison, WI, USA), 2.5 μL of

Table 1. The RAPD decamer primers and their sequences used for the genetic fidelity evaluation

Primer name	Sequences 3'-5'	Primer name	Sequences 3'-5'
OPA 01	CAGGCCCTTC	OPA 09	GGGTAACGCC
OPA 03	AGTCAGCCAC	OPA 11	CAATCGCCGT
OPA 04	AATCGCGCTG	OPB 09	TGGGGGACTC
OPB 11	GTAGACCCGT	OPB 17	AGGGAACGAG
OPAB 11	GTGCGCAATG	OPB 18	CCACAGCAGT
OPAL 20	GAACCTGCGG	OPC 02	GTGAGGCGTC
OPE 14	TGCGGCTGAG	OPC 14	TGCCTGCTTG
OPH 02	TCGGACGTGA	OPD 20	ACCCGGTCAC
OPG 07	GAACCTGCGG	OPF 02	GAGGATCCCT
OPC 14	TGCCTGCTTG	OPF 13	GGCTGCAGAA

The primers in bold generated amplification products in all the analysed samples

MgCl$_2$ (Promega Corp., Madison, USA), 0.5 μL of a dNTP mix (Promega Corp., Madison, USA), 0.5 μL of a RAPD primer (Microsynth, Balgach, Switzerland), and 0.2 μL of a GoTaq polymerase (Promega Corp., Madison, WI, USA). The DNA amplification was carried out in a 96 Well Gradient Palm-Cycler CG1-96 (Corbett Research, Sydney, Australia) programmed for 1 cycle of 3 min at 95 °C, followed by 45 cycles of 1 min at 93 °C, 1 min at 34 °C and 1 min at 72 °C. After a final incubation for 10 min at 72 °C the samples were stored at 4 °C prior to analysis.

For the SRAP analysis, the PCR reactions were carried out following the protocol described by Li and Quiros (2001), but the reaction volumes were adjusted to 15 μL. The reaction mixtures (a total volume of 15 μL) consisted of 50 ng of genomic DNA, 0.3 μM of each primer (Kaneka-Eurogentec, Belgium), 1.5 mM of MgCl$_2$, 0.2 mM of dNTPs, 5X of Green PCR buffer, 1 U of GoTaq DNA polymerase (Promega, USA) and nuclease-free water (Sigma-Aldrich GmbH, Germany). The DNA amplification was carried out in a 96 Well Gradient Palm-Cycler CG1-96 (Corbett Research, Sydney, Australia):

5 min of denaturation at 94 °C, five cycles of 1 min of denaturation at 94 °C, 1 min of annealing at 35 °C and 1 min of elongation at 72 °C and then 35 cycles (94 °C for 1 min; 50 °C for 1 min and 72 °C for 1 min) with a final elongation step of 10 min at 72 °C.

The PCR amplifications were repeated twice for each RAPD primer and SRAP primer combinations to ensure the reproducibility of the results. Separation of the amplified products for both of the used PCR based techniques was performed by electrophoresis on 2% agarose gels with ethidium bromide detection. The electrophoretic profiles were visualised under UV (ultraviolet) light and the images were captured by BioSpectrum AC Imaging System. The gel images were analysed using TL120 software (Nonlinear Dynamics, Newcastle upon Tyne, UK) to determine the range of the molecular weight (bp) of the RAPD and SRAP amplified bands.

RESULTS AND DISCUSSION

Both blackberry cultivars had a high average multiplication rate over 40 (data not shown) on the

Table 2. The SRAP primers used in the present study

Primer	Sequences 3'–5'	Primer	Sequences 3'–5'
Me*1	TGAGTCCAAACCGGATA	Em3	GACTGCGTACGAATTGAC
Me4	TGAGTCCAAACCGGACC	Em4	GACTGCGTACGAATTTGA
Me6	TGAGTCCAAACCGGTAA	Em5	GACTGCGTACGAATTAAC
Me8	TGAGTCCAAACCGGTGC	Em6	GACTGCGTACGAATTGCA
Em**1	GACTGCGTACGAATTAAT	Em7	GACTGCGTACGAATTCAA
Em2	GACTGCGTACGAATTTGC	Em8	GACTGCGTACGAATTCTG

Me* and Em** represent the forward and reverse primers, respectively

Table 3. The number and size range (bp) of the RAPD amplified band

Primer name	Range of molecular weight of amplified bands (bp)	Number of amplified bands 'Loch Ness'/'Chester'
OPA-01	650–1 065	3/4
OPB-11	490–1 160	5/4
OPB-18	375–1 265	5/5
OPAB-11	624–1 745	4/3
OPC-14	296–1 624	5/6
OPF-02	420–1 675	4/3
OPE-14	455–1 850	5/4
OPAL-20	417–965	3/4
Total		33/33

low-cost MSm medium used in this experiment. The average multiplication rate was calculated after 10 weeks of culture from 5 vessels (5 explants/vessel) by averaging the total number of shoots induced per explant. It was observed that the highest proliferation rates were reached in the case of the 2 cm length explants with at least 3 buds inoculated in the culture media. Besides the cost effectiveness of this medium, another advantage of its use was that no hyperhydrated, vitreous plants had developed during the *in vitro* culture. Along the ten subcultures, neither the multiplication rate was decreased nor any morphological anomalies of the shoot or the leaves were observed.

RAPD analysis. In total, 20 RAPD primers were used for the initial screening between the mother plant and the clones that resulted from the micropropagation of both of the analysed blackberry cultivars, 'Loch Ness' and 'Chester', but only eight RAPD primers gave clear and reproducible bands. The number of scorable bands for each RAPD primer varied from 3 (OPA -01; OPAL -20) to 5 (OPB-11; OPB-18; OPC 14) (Table 3).

The 8 RAPD primers generated 33 distinct and scorable bands for each of the two cultivars, with an average of 4.12 bands per primer. Each RAPD primer generated amplification products ranging in size from 375 bp (OPB-18) to 1850 bp (OPE-14). Our results show that the primers produced amplification products, which were monomorphic across all the micropropagated plants and no polymorphism was detected during the RAPD analysis of the *in vitro*-raised clones (Figures 1 and 2). The micropropagated plants derived from shoot tips and axillary buds have been previously reported to maintain genetic stability (Ostray et al. 1994; Samantaray, Maiti 2010). An explanation for these results may be that the genetic stability is maintained when the meristem culture is used for micropropagation purposes because organised meristems are generally more resistant to the genetic changes that might occur during the cell division and the *in vitro* differentiation (Shenoy, Vasil 1992).

SRAP analysis. Our results show that SRAP markers were suitable to assess the genetic fidelity of the analysed blackberry cultivars. A total of 32 SRAP primers were employed for the genetic fidelity analysis of the plants derived from the *in vitro* shoot culture, but only 12 primer combinations produced amplification patterns which are identical with the mother plants (Table 4).

As it can be noticed in Table 4, the smallest number of bands was recorded in the 'Loch Ness' (59) cultivar when compared to the 'Chester' (55) one,
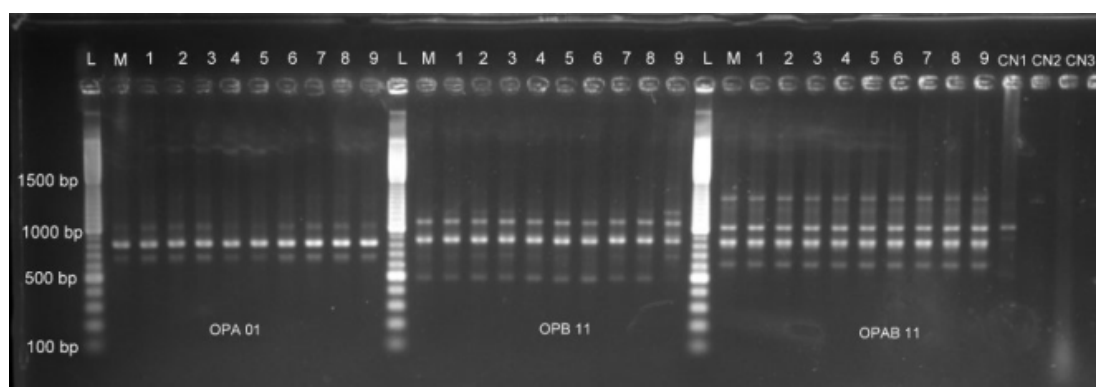

Figure 1. The RAPD profiles of the mother plant and the micropropagated clones of the 'Loch Ness' cultivar generated by primers OPA-01, OPB-11 and OPAB-11, respectively

Lanes: M – RAPD bands from the mother plant; 1–9 – sub-cultured plants; L – molecular marker (100 bp Ladder, Promega, USA); CN1–CN3 – sample controls without DNA

Table 4. The total number of bands, monomorphic bands and the size range (bp) of the SRAP amplified bands

Primer combination	Size range of bands (bp)	Number of total bands 'Loch Ness'/'Chester'	Number of monomorphic bands 'Loch Ness'/'Chester
Me1-Em3	240–990	3/4	3/4
Me1-Em4	370–1 250	4/4	4/4
Me1-Em2	290–1 460	6/7	6/7
Me1-Em8	310–1 300	5/5	5/5
Me4-Em4	270–1 560	6/7	6/7
Me4-Em5	170–1 450	7/8	7/8
Me4-Em6	220–1 190	6/6	6/6
Me6-Em6	300–1 230	6/5	6/5
Me6-Em7	210–1 700	5/7	5/7
Me8-Em1	340–960	4/4	4/4
Me8/Em7	220–780	3/4	3/4
Me8-Em8	330–1 260	4/4	4/4
Total		59/55	59/55

giving proof that the two cultivars under study had different SRAP profiles, despite the fact that no differences were shown between the mother plants and their clones obtained through the micropropagation.

The results of our study confirm that the SRAP-PCR markers can be a rapid evaluation of the genetic stability for the *in vitro* generated plants at any stage of micropropagation (Figure 3). In addition, SRAP markers can be used to assess the genetic stability of the blackberry plantlets in each stage of the *in vitro* culture. According to previous studies (Sun et al. 2014; Li et al. 2014), SRAP-PCR markers are suitable to assess the genetic fidelity of the tissue culture plants; whose results are in accordance with our findings. Other reports reveal that no genetic variability was detected in the *in vitro* culture plants of *Oci-*

mum basilicum, suggesting that nodal explants can be successfully used for the *in vitro* multiplication of *O. basilicum* on a large scale without the risk of the genetic instability of the plantlets (Saha et al. 2014).

Our results show that both the RAPD and SRAP marker systems are useful for cultivar differentiation. Thus, in the RAPD electrophoretic profiles for 'Loch Ness', 3 amplification products were identified and ranged in length from 600 to 1050 bp (OPA 01), while 4 amplification products were identified (OPA 01) in 'Chester'. A similar differentiation was obtained between the cultivars by the SRAP markers as well, where 7 amplification products were identified in 'Loch Ness' with primers Me4/Em5, ranging from 250 and 1 300 bp while 8 amplification bands were identified in the range of 150–1200 bp (Me4/Em5) in
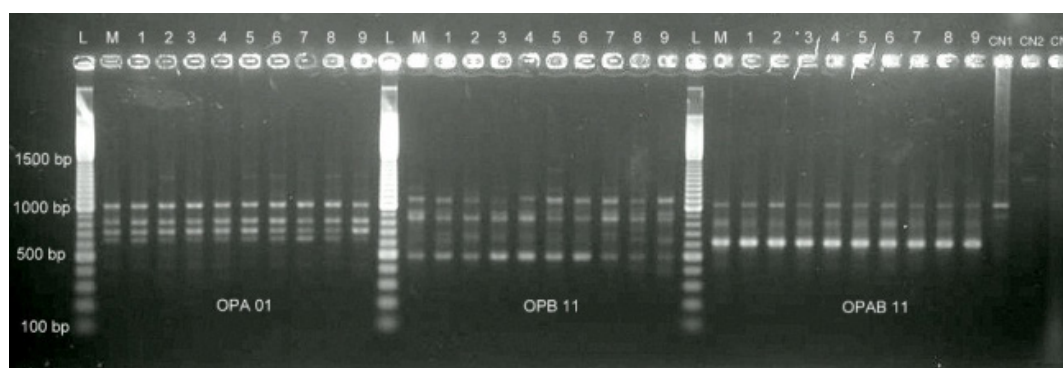

Figure 2. The RAPD profiles of the mother plant and the micropropagated clones of the 'Chester' cultivar generated by primers OPA-01, OPB-11 and OPAB-11, respectively

Lanes: M – the RAPD bands from the mother plant; 1–9 –sub-cultured plants. L – molecular marker (100 bp Ladder, Promega, USA); CN1–CN3 – sample controls without DNA
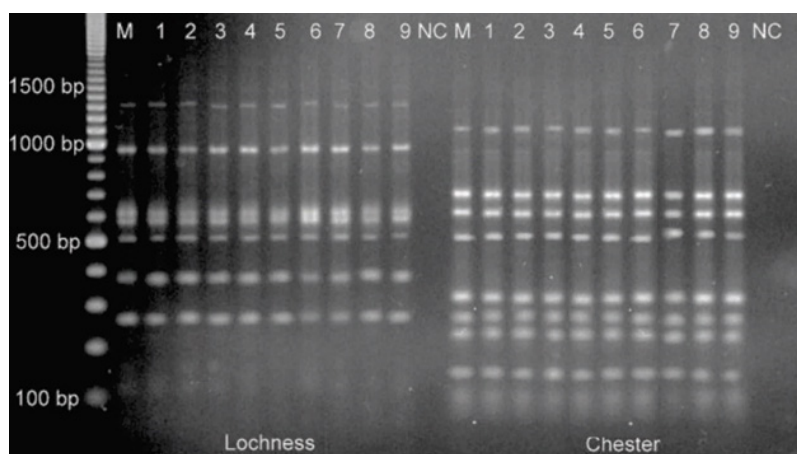
Figure 3. The monomorphic SRAP profiles of the mother plant and the micropropagated clones of the 'Loch Ness and 'Chester' cultivars generated by the primer combination Me4-Em5

Lanes: M – SRAP bands from the mother plant; 1–9 – sub-cultured plants; ladder – indicates the molecular marker (100 bp Ladder, Promega, USA); NC – sample controls without DNA

'Chester'. According to a previous study, the RAPD analysis detected a high genetic differentiation among the *Olea europaea* L. cultivars as reported by Belaj et al. (2002).

The conclusions that emerged from the foregoing observations are that the micropropagation protocol used in this experiment is efficient and profitable for the mass propagation of *Rubus fruticosus* L. and both the RAPD and SRAP markers can successfully be used to assess the genetic fidelity in the micropropagated plants that resulted from the 'Loch Ness' and 'Chester' cultivars.

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