Micropropagation and Assessment of Genetic Stability of Acclimatized *Streptocarpus* x *hybridus* Voss Plantlets Using RAPD Markers

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Abstract

A micropropagation protocol via direct shoot organogenesis from *Streptocarpus* x *hybridus* Voss. leaf explants was established in this study. The shoot induction of three *Streptocarpus* cultivars ('Snow White', 'Black Panther' and 'Slumber Song') was successfully achieved on Murashige and Skoog (MS) medium supplemented with 0.2 mg L^{-1} -indole-3-acetic acid (IAA) and 0.2 mg L^{-1} thidiazuron (TDZ). In proliferation stage, the effects of two combinations of plant growth regulators -PGR- (V1-0.2 mg/L^{-1} IAA + 0.5 mg/L^{-1} BAP and V2-1.0 mg L^{-1} NAA + 0.2 mg L^{-1} TDZ) on shoot number and length were examined. The results suggest that PGRs combinations significantly influenced shoot proliferation and root induction in all *Streptocarpus* cultivars. Among the treatments, 0.2 mg L^{-1} (IAA) in combination with 0.5 mg L^{-1} 6-benzylaminopurine (BAP) were the most effective for *in vitro* shoot multiplication and rooting. The *in vitro* rooting percentage was also determined before subjecting the plantlets to the acclimatization process. Due to acclimatization, *Streptocarpus* plantlets showed a very high rate of survival (90%). The generated PCR-RAPD profiles for the selected *in vitro*-raised plants and donor plants were similar which indicates the clonal or true-to-type nature of the progenies.

Keywords: Cape primrose, clones, donor plant, genetic fidelity, monomorphism

Introduction

Streptocarpus x *hybridus*, commonly known as Cape primrose, is belonging to the *Gesneriaceae* family and is extensively cultivated worldwide as a flowering houseplant because of its aesthetic value given by attractive colors of the flowers (Chaudhury *et al*., 2010).

It is worth mentioning that acaulescent species of *Streptocarpus* subgenus, including the *S.* x *hybridus* do not develop the leaves on a stem. The leaf lamina have a continuous growth from a basal meristem and a stemlike petiole (Möller and Cronk, 2001; Nishii and Nagata, 2007; Edwards *et al*., 2008). Moreover, in young plants, the flowers arise from the upper part of the midrib of the leaf (Appelgren and Heide, 1972) and are grouped in a cyme inflorescence with two-six zygomorphic tubular flowers on an upright peduncle (Hilliard and Burt, 1971). Much hybridising work has been done to produce the current modern hybrids with a widened range of color and shape of the flowers and also with differences on the plant size. The most *Streptocarpus* x *hybridus* varieties are crosses between different species that have been

Figure 1. *Streptocarpus x hybridus* V. varieties used as donor plants for *in vitro* multiplication (A-'Snow White'; B- 'Black Panther'; C- 'Slumber Song')

cultivated first in the early 1800s in South Africa (Marston, 1964, Afkhami-Sarvestani *et al*., 2012). Commercial cultivars that have been obtained through artificial hybridization and induced mutagenesis have a compact rosette growth and a considerably reduced leaf size (15 - 20 cm) and usually exhibit a high variability regarding shape, size, scent and flower color, which define each variety in particular (Cantor *et al*., 2004).

In order to propagate the hybrids and keep them identical to the genitor plants vegetative propagation methods must be applied. In this context, micropropagation through tissue culture techniques is one of the most important approach to ornamental plant propagation on a large-scale (Jain *et al*., 2006).

In vitro shoot proliferation is usually used for clonal mass propagation of a specific genotype and year-round production of plants. However, the environmental conditions of *in vitro* culture and its duration may induce some somaclonal variations in micropropagated plants which can lead to a reduced commercial value (Debnath *et al*., 2012; Krishna *et al*., 2016). The occurrence of somaclonal variation is a potential drawback when clonal fidelity is required (Naseer *et al*., 2016). Moreover, some genetic and biochemical changes which appear at molecular level during *in vitro* culture can be expressed at the phenotypic level, but sometimes may not be clearly identified and evaluated in the young plants acclimatized under *ex vitro* conditions (Palombi and Damiano, 2002). Therefore, molecular markers represent a valuable tool for genetic stability assessment of micropropagated plants and out of which RAPDs can successfully be used for genetic fidelity studies of *in vitro*-raised plants (Handa, 1998; Martin *et al*., 2002; Kawiak and Lojkowska, 2004; Santos *et al*., 2008; Senapati *et al*., 2012; Jiang, 2014; Ilczuk and Jacygrad, 2016).

There have been a few reports on the propagation of *S.* x *hybridus* using tissue culture technologies (Peck and Cumming, 1984; Cantor *et al*., 2004; Chaudhury *et al*., 2010; North and Ndakidemi, 2012) but to the best of our knowledge, this is the first report to refer to the study of genetic stability of the micropropagated *S*. x *hybridus* progenies.

The aim of this study was to evaluate the *in vitro* propagation protocol used for the regeneration of three *Streptocarpus* cultivars and to assess the genetic identity of the newly obtained *in vitro* plantlets as compared to the mother plants. Testing the genetic homogeneity of *in vitro*-raised plants adds value to any technology that is being developed for trading purposes. The survival rate of the *in vitro* regenerated plantlets was also determined after acclimatization process.

Materials and Methods

Plant material

In the present study, three *Streptocarpus* x *hybridus* V. cultivars were used for *in vitro* micropropagation. The mother plants have been selected based on flower characteristics and were purchased from two different certified nurseries from North Wales, UK. 'Snow White' is a miniature variety from 'Maassen's White' with more tubular flowers, white corolla and almost erect calyx. 'Black Panther' is an American variety created by J. Ford with dark purple flowers and two thin yellow bars from the throat of each flower. 'Slumber Song' is another variety created also by J. Ford, with medium purple color of the corolla, pink splashes on all petal lobes and strong yellow throat (http://www.streptocarpus-info.com).

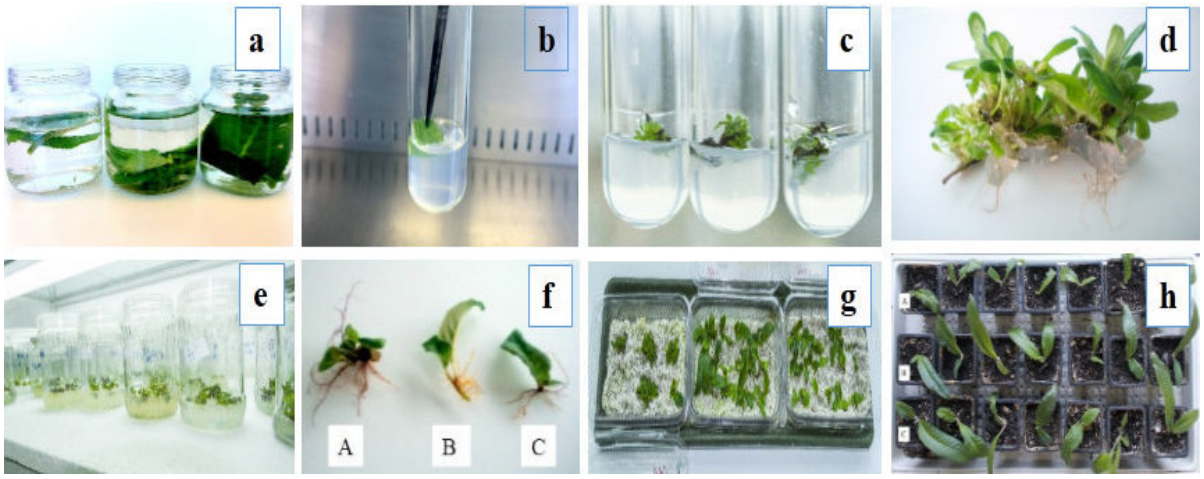

Figure 2. *In vitro* shoot multiplication of *Streptocarus* x *hybridus* V. cultivars (A-'Snow White'; B-'Black Panther'; C-'Slumber Song'). Sterilization of the leaf explants (*a*); leaf fragment inoculation under aseptic conditions (*b*); shoot induction from leaf explants (*c*); adventitious shoot proliferation (*d*); shoot multiplication in growth room (*e*); rooted plantlets acclimatization in perlite (*f-g*) hardening of the plants in greenhouse conditions (*h*)

All plant materials used in this research for tissue culture initiation were previously grown as potted plants in greenhouse conditions at 23 ºC (Fig. 1).

In vitro shoot induction and plant regeneration

To initiate *in vitro* culture, young leaves from mother plants were collected and washed gently in running tap water for 3 min to remove any surface impurities (Fig. 2*a*).

The leaves were then cut into fragments (10x10 mm) and were sterilized with 20 % commercial bleach ACE solution (Procter & Gamble, Romania; <5 % active ingredient) for 20 min. After sterilization, the explants were rinsed three times with sterile deionized water and were aseptically inoculated in test tubes (5 cm×9.5 cm) containing Murashige and Skoog (1962) medium (MS) supplemented with 2 % (w/v) sucrose, 0.2 mg L^{-1} IAA and 0.2 mg L^{-1} TDZ and solidified with 0.6% agar (Sigma-Aldrich Inc.) (Fig. 2*b*). The pH of culture medium was adjusted to 5.9 before autoclaving at 121˚C for 20 min. The cultures were grown in culture room at 24±1°C under 16/8 h light/dark cycle. Light was provided by white fluorescent lamps.

Eight weeks after inoculation, the proliferated cluster of shoots was divided and the *in vitro* shoots were individually transferred to culture jars containing fresh MS medium supplemented with two different combinations of plant growth regulators (PGRs): 0.2 mgL^{-1} IAA and 0.5 mgL^{-1} BAP (V1) and 1.0 mgL^{-1} NAA and 0.2 mgL^{-1} TDZ (V2), respectively. Adventitious shoots were separated (after 8-weeks) and subsequently transferred aseptically to culture jars and subcultured at 24 ± 1°C under fluorescent white light (33,6 µmol m-2 s-1) conditions with a photoperiod of 16/8 h light and dark cycles (Fig. 2*d-e*). The influence of two experimental factors (PGRs combination x cultivar) on the number of shoots/explant, shoots length/explant and *in vitro* rooting percentage (%) were also examined and measured after the 12^{th} subculture.

After the 12^{th} subculture the regenerated plantlets were subjected to acclimatization process. For this, the *in vitro* plantlets with well-developed rooting systems were taken out from the culture jars and rinsed carefully with sterile deionized water to eliminate the remaining solidified agar medium from the roots (Fig. 2*f*). The cleaned plantlets were then planted in transparent plastic containers with lids filled up with humidified perlite (Fig. 2*g*). The plastic containers were covered and then placed into the acclimatization room at 24 ± 1°C where the plantlets were exposed to natural light conditions and frequent hand-misting. The percentage of the acclimatized plantlets was determined after 4 weeks and followed by the transplantation of the plantlets to plastic containers with a diameter of 6 cm filled with a potting mixture made of peat, vermiculite and perlite (1:1:1). The potted plants were irrigated with tap water at every 4 days and placed into a growing chamber providing favorable growth conditions according to the plant's specific environmental requirements for growth as described by Cantor et al. (2004). The

Table 1. Primer sequences and the number of scored monomorphic bands generated by RAPD markers in *Streptocarpus x hybridus* V. cultivars

Primer name	Sequences 5'-3'	No. of recorded bands			Primer name	Sequences 5'-3'	No. of recorded bands		
		SW	BP	SS			SW	BP	SS
OPA 01	CAGGCCCTTC	-	-	-	OPA 09	GGGTAACGCC	-	-	-
OPA 03	AGTCAGCCAC	4	6	4	**OPA 11**	CAATCGCCGT	4	6	6
OPA 04	AATCGCGCTG	-	-	-	**OPB 08**	GTCCACACGG	5	4	8
OPB 11	GTAGACCCGT	5	4	8	OPB 17	AGGGAACGAG	-	-	-
OPAB 11	GTGCGCAATG	4	6	6	**OPB 18**	CCACAGCAGT	4	6	7
OPAL 20	GAACCTGCGG	6	7	6	OPC 02	GTGAGGCGTC	-	-	-
OPE 14	TGCGGCTGAG	-	-	-	OPC 14	TGCCTGCTTG	-	-	-
OPA 17	GACCGCTTGT	-	-	-	OPD 04	TCTGGTGAGG	-	-	-
OPH 02	TCGGACGTGA	-	-	-	**OPD 20**	ACCCGGTCAC	5	6	6
OPG 07	GAACCTGCGG	5	4	7	**OPF 02**	GAGGATCCCT	5	7	8
OPC 14	TGCCTGCTTG	-	-	-	OPF 13	GGCTGCAGAA	-	-	-
OPF 12	ACGGTACCAG	-	-	-	OPH 12	ACGCGCATGT	-	-	-
Total monomorphic bands		24	27	31			23	29	35
Average number/primer		4.7	5.6	6.6					

*Bolded primers generated amplification products in all the analyzed samples. The number of monomorphic bands was scored for each variety ('Snow White'-SW, 'Black Panther'-BP and 'Slumber Song'-SS)

survival rate of potted plants was recorded after the 8th week.

DNA extraction and PCR amplification

Fresh young *Streptocarpus* leaf samples were collected from mother plants (potted-grown plants used as explant source for *in vitro* culture initiation) and also from the randomly selected acclimatized plantlets (7 plants/each cultivar) (Fig 2*h*). The DNA was isolated using a CTAB-based protocol published by Lodhi *et al*. (1994) and improved by Pop *et al*. (2003) and Bodea *et al*. (2016). PCR amplification reactions were carried out as described by Williams *et al*. (1990). Reaction mixtures (25 µL total volume) consisted of 50 ng DNA, 9.3 µL distilled H_20 for PCR reactions, 2 µL PVP (polyvinylpyrrolidone), 5 µL GoTaq Flexi green buffer (Promega Corp., Madison, WI, USA), 2.5 µL MgCl2 (Promega Corp., Madison, WI, USA), 0.5 µL dNTP mix (Promega Corp., Madison, WI, USA), 0.5 µL RAPD primer (Microsynth, Balgach, Switzerland), 0.2 µL GoTaq polymerase (Promega Corp., Madison, WI, USA). DNA amplification was carried out in a 96 Well Gradient Palm-Cycler CG1-96 (Corbett Research, Sydney, Australia) set for 1 cycle of 3 min at 95 ºC, followed by 45 cycles of 1 min at 93 ºC, 1 min at 34 ºC and 1 min at 72 ºC. After the final extension for 10 min at 72 ºC the samples were stored at 4 ºC prior to analysis. The RAPD amplified products were size fractionated by electrophoretic migration on a 1.2% agarose (Sigma-Aldrich) gel in 1X TAE Buffer at 0.29 V/cm2 for 2 hours. The molecular marker used was 100bp DNA Step Ladder (Promega Corp., Madison, WI, USA). For visualization of the DNA profiles, the gels were stained with 0.5 µg/µl Ethidium Bromide for 25 min and then examined under UV light Biospectrum AC Imaging System (UVP BioImaging Systems, Upland, CA).

In order to perform RAPD analysis, 24 decamer primers were used, but only ten yielded scorable amplification patterns for all the analyzed samples (Tab. 1).

Data analysis

The experiments were carried out in a completely randomized design. For each *Streptocarpus* cultivar, seven culture jars with seven proliferated explants (7x7inoculums) were evaluated for shoot multiplication. The *in vitro* rooting percentage was calculated before subjecting the *in vitro* plantlets to acclimatization. One-way ANOVA was used to assess the statistically significant differences between the data recorded.

Table 2. Effect of plant growth regulator (PGR) combinations on the proliferation and *in vitro* rooting of *Streptocarpus x hybridus* V. shoots

PGR combination	V1 0.2 mgL^{-1} IAA +0.5 mgL^{-1} BAP			V2 1.0 mgL^{-1} NAA + 0.2 mgL^{-1} TDZ		
Cultivars	No. of shoots/ explant	Length of shoots (cm)	Rooting (%)	No. of shoots/ explant	Length of shoots (cm)	Rooting (%)
'Snow White'	5.03±0.11bC*	2.67±0.02bA	80.70±0.17bA	4.50±0.08aC	2.16±0.01aA	70.35±0.22aA
'Black Panther'	3.05±0.07bA	4.01±0.03bB	82.83±0.22bB	2.64±0.07aA	3.54±0.02aB	73.73±0.18aB
'Slumber Song'	3.63±0.07bB	4.51±0.02bC	83.12±0.09bB	3.10±0.06aB	3.95±0.03aC	73.87±0.22aB

The values shown are means ±SE. *Different lowercase letters indicate significant differences between the treatments and different capital letters indicate significant differences among the cultivars subjected to the same treatment, according to Duncan's test.

Statistically significant differences between the means were checked by applying Duncan's multiple range test ($p<0.5$) using SPSS v.16. The values shown are means ±SE.

For molecular analysis, gel images were analyzed using TotalLab TL120 software (Nonlinear Dynamics, Newcastle upon Tyne, UK). Only distinct and reproducible bands of the electrophoretic profiles generated by selected RAPD marker combination were scored as 1 (present) or 0 (absent) and transferred to a binary matrix using MS Excel. Genetic distances were calculated using the Nei's (1972) similarity coefficient based on the data obtained. An UPGMA (Sneath and Sokal 1973) dendrogram was also constructed based on a symmetric dissimilarity matrix using the SHAN module in NTSYS program, version 2.1 (Rohlf, 2000).

Results

Effects of PGRs on shoots proliferation and root development

In this study, MS medium with two different combinations of plant growth regulators were tested to choose the best culture medium. The PGR combinations significantly affected shoot proliferation in all *Streptocarpus* cultivars (Tab. 2). Among the treatments, 0.2 mg L^{-1} AIA in combination with BAP (0.5 mg L^{-1}) proved to be the best for shoot multiplication (Table 2). The largest shoot number per explant (5.03) was recorded in 'Snow White' while the smallest (2.64) was observed in 'Black Panther' cultured on MS medium supplemented with 1.0 mg L^{-1} NAA and 0.5 mg L^{-1} TDZ.

The data also show that the longest shoots were recorded on MS medium containing V1-PGRs combination ('Slumber Song' and 'Black Panther') while the shortest (2.16 cm) were recorded on the medium containing V2 -PGR combination ('Snow White'). Significant differences were observed between the three cultivars cultured on the same PGR combination. It was noticed that 'Snow White' produced the highest number of shoots/ explant but with the smallest mean length of the proliferated shoots (Tab. 2). The shoots in 'Black Panther' and 'Slumber Song' were significantly longer as compared to 'Snow White' on both culture medium with the two PGR combinations (V1 and V2) as shown in Table 2.

In vitro rooting of multiple shoots was recorded on both V1 and V2 culture medium for all *Streptocarpus* cultivars. The rooting percentage of the shoots is presented in Table 2. The results show that MS medium supplemented with 0.2 mgL^{-1} IAA and 0.5 mgL^{-1} BAP stimulated mostly the root formation of the explants (83.12%, 82.83% and 80.70%) as compared to the results obtained in the same varieties grown on MS medium supplemented with 1.0 mgL^{-1} NAA and 0.2 mgL^{-1} TDZ (73.87, 73.73 and 70.35%). Furthermore, significant differences were also recorded between 'Snow White' and the other two cultivars 'Black Panther' versus 'Slumber Song' as summarized in Table 2.

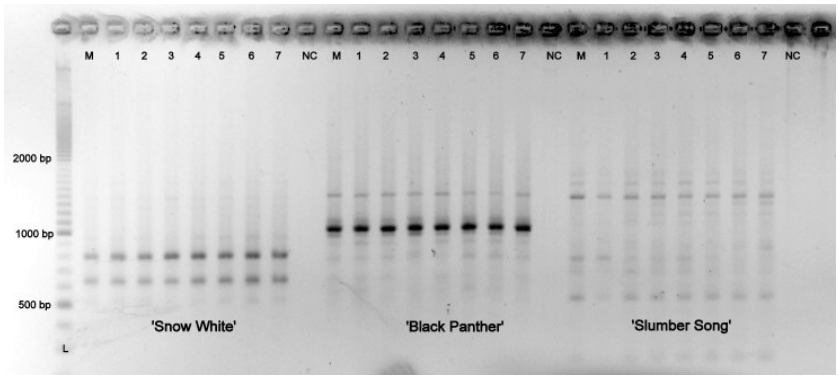

Figure 3. RAPD profile of *Streptocarpus* plantlets obtained with OPF 02 primer. (Lane L-100 bp DNA step Ladder; M-mother plant for each cultivar; L1-L7 acclimatized plantlets; NC-negativ control)

Acclimatization of plantlets to ex vitro conditions

Regarding the acclimatization process, it is noteworthy that almost 90% of the *in vitro* plantlets from the three cultivars survived and showed normal growth and development. Following to the acclimatization process, as mentioned before in Materials and methods, potted plants were irrigated with tap water at every 4 days and successfully hardened as houseplants with 70% survival rate after the 8th week.

Assessment of genetic stability of acclimatized plants

In order to confirm whether somaclonal variation was detectable or not in the regenerated plants, RAPD was employed to analyze the genetic fidelity of seven plants from each cultivar from the randomly selected *in vitro* plants, as well as their corresponding mother plants. From the 24 initially screened RAPD primers, 10 produced clear and scorable bands for all the analyzed samples. The RAPD marker analysis resulted in a total of 47, 56 and 66 monomorphic bands for 'Snow White', 'Black Panther' and 'Slumber Song', respectively (Tab. 1). Figure 3 shows that the *in vitro* derived plants shared the same banding patterns as those of the donor plants indicating their possible genetic similarity.

No polymorphic bands were obtained in any of the analyzed cultivars. Each RAPD markers generated an average of 4.7, 5.6 and 6.6 bands for the analyzed cultivars (Tab. 1) and their length ranged between 580 and 1840 bp.

Discussion

Our results suggest that the protocol used for *in vitro* culture of *S. x hybridus* cultivars was suitable for shoot induction, proliferation and *in vitro* rooting induction. The combination of 0.2 mgL^{-1} IAA and 0.5 mgL^{-1} BAP was found to be effective to produce more multiple rooted shoots than the one with NAA and TDZ. These results are consistent with those reported by Chaudhury *et al*. (2010) who found that the inclusion of BAP at 0.5 mgL^{-1} in the regeneration medium had a significant influence on shoot proliferation from leaf explants of *Streptocarpus* x *hybridus* varieties namely 'Amanda', 'Branwen' and 'Chorus Line'. Similarly, our results match those observed in earlier studies by North and Ndakidemi (2012) who have employed 0.5 mgL^{-1} BAP and 0.1 mgL^{-1} IAA on MS medium for optimal proliferation of multiple shoots of *Streptocarpus nobilis*.

Efficient *in vitro* rooting method development is also important for a better regeneration of *Streptocarpus* plants. Therefore the use of auxins and cytokinins may have a decisive role in bud and root formation of *in vitro* plants as stimulators or inhibitors depending on the concentration ratio applied. Similar studies showed that both bud and root formation can be stimulated by auxins, but the optimum for root development being up to two fold higher than for bud formation (Appelgren and Heide, 1972). Our findings confirm that auxins have an important role also in *Streptocarpus* tissue culture development as stimulators of root formation. Similar results have been reported in other plant species as well (Soliman *et al*., 2014). Regarding cytokinins, if they are not used in theoptimum concentration in combination with auxins, at low concentrations they can have an insignificant stimulatory effect but in higher concentrations they can inhibit or antagonize

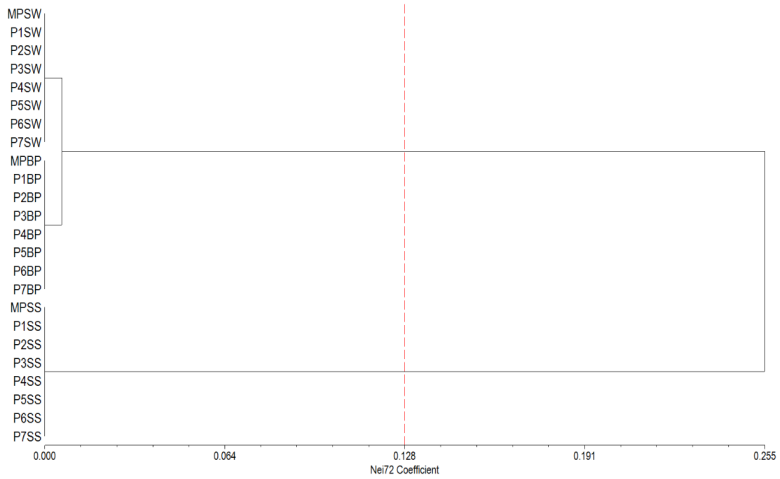

Figure 4. Dendrogram of the *Streptocarpus* plantlets and mother plants derived from UPGMA cluster analysis based on Nei72' coefficient

the stimulatory effect of auxins on plant organ formation.

The findings observed in this study (summarized in Tab. 2) are in agreement with those reported by Cantor *et al.,* 2004 that have examined the effect of 1.0 mgL^{-1} NAA (1 naphthaleneacetic acid) and 0.5 mgL^{-1} TDZ (thidiazuron) added to the MS medium on *Streptocarpus* x *hybridus* regeneration resulting in a high frequency (70-100%).

The efficiency of the protocol presented in this study consists in shoot induction and direct plant regeneration devoid of callus phase. Moreover, the clusters of *in vitro* proliferated shoots formed normally developed roots. These plantlets were then individually separated and acclimatized with a high rate of survival (90%). Therefore, production of adventitious rooted shoots from leaf explants in *Streptocarpus* x *hybridus* is useful because direct regenerated plants may exhibit greater genetic stability than those produced *via* callus (Sarmast *et al*., 2012).

Despite the many advantages of micropropagation through tissue culture, the presence of somaclonal variation is a major problem encountered in the *in vitro* culture (Soliman *et al*., 2014). Factors that can cause somaclonal variations during *in vitro* culturing may include chromosomal rearrangements, changes at molecular level, such as single-nucleotide changes, deamplification and amplification of genes, alterations in DNA methylation patterns and transposable element activations (Ilczuk and Jacygrad, 2016). Likewise, the combination and concentrations of plant growth regulators, culture length, period and method of regeneration may also influence the genetic stability of the *in vitro* regenerated plantlets (Martins *et al*. 2004). Even at optimal levels, long term *in vitro* cultures may often lead to somaclonal or epigenetic variations in micropropagated plants (Saha *et al*., 2016). Even so, no genetic variation was observed among mother plants (MP) and the seven randomly selected clones (P1–P7) from each acclimatized cultivar as recorded after 12^{th} subculture (Fig. 4).

Our results support previous findings reported by Varshney *et al*. (2001) who revealed that the RAPD patterns produced to *Lilium* sp. (Asiatic hybrids) were all shared by both *in vitro*-raised bulblets (randomly selected after four and 12 subcultures) and mother bulbs.

Our results further suggest that the RAPD molecular marker approach is a useful tool in the evaluation of the genetic stability of *in vitro* propagated *Streptocarpus* x *hybridus* V. plants**.**

Conclusion

As a conclusion, the micropropagation protocol was efficient consisting of shoot induction and direct plant regeneration in devoid of the callus phase. Besides, the production of adventitious rooted shoots from of *Streptocarpus* x *hybridus* leaf explants is useful considering that direct regenerated plants may exhibit greater genetic stability than those produced via callus. Therefore, the propagation method presented here may be applied for commercial purposes as well.

The results of this investigation also show that the micropropagated plantlets are likely to be true to type of the mother plants as no polymorphic bands were identified in the randomly selected *in vitro* derived plants and their corresponding mother plants during the RAPD analysis.

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