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Protoplast Isolation and Culture from *Streptocarpus*, Followed by Fusion with *Saintpaulia ionantha* Protoplasts

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Summary

To produce new ornamental plants by combining *Saintpaulia ionantha* and *Streptocarpus,* two closely related genera of the family *Gesneriaceae,* protoplast fusions were accomplished. Various tissues were evaluated as potential starting materials for protoplast isolation from *Streptocarpus saxorum* × *S. stomandrus* and *S. caulescens*. Sufficient protoplast yields between 2 and 3.6×10^5 g^{-1} fresh mass with high viabilities from 68–96 % were obtained from the leaves of in vitro shoot cultures, which were grown in liquid medium in the dark. After 14 days of culture, cell divisions reached frequencies between 2.1 and 5.5 %, depending on the species, and vigorously growing calluses were obtained. However, only root formation was induced on these calluses; no shoot induction was achieved.

For the protoplast fusion, polyethylene glycol was applied and an appropriate fusion protocol was developed. The fusion rate, which was determined by cell nucleus staining with ethidium bromide, reached up to 35 % binuclear protoplasts. Cell divisions were observed in four fusion experiments between two *Streptocarpus* species and two *Saintpaulia ionantha* varieties, and in two of these experiments, callus formation was also observed.

To identify possible hybrid calluses, calluses were preselected by flow cytometry and analysed with RAPD markers. In 27 out of 30 tetraploid calluses the DNA bands from the *Streptocarpus* parent were dominant or were the only ones detected, in one the bands from the *Saintpaulia* parent, and only two calluses with bands of both parents were observed. This evidence supports the notion that successful fusions between *Saintpaulia* and *Streptocarpus* are possible, but no plants could be regenerated from the hybrid calluses.

Key words. callus formation – ornamental plants – protoplast isolation – polyethylene glycol (PEG) – somatic hybridisation

Introduction

Within the *Gesneriaceae* family, two genera contain species that are commercially used as ornamental plants, namely *Saintpaulia ionantha* and the *Streptocarpus* species and their hybrids. Interestingly, *Saintpaulia* and members of the *Streptocarpus* subgenus *Streptocarpella* were found to be closely related as indicated by pollen structure (Holmqvist 1964), exine scalping (Weigend and Edwards 1996), chromosome number (x = 15, Skog 1984) and molecular phylogenetic studies (Jong 1973; Möller and Cronk 1997, 1999, 2001a, b; Smith et al. 1998; Möller et al. 1999; Jong and Möller 1999; Hughes et al. 2004). Möller and Cronk (1997), using the internal transcribed spaces (ITS), showed that the genus *Saintpaulia* evolved from within the subgenus *Streptocarpella* of the genus *Streptocarpus*.

Breeding of the African violet (*Saintpaulia ionantha*) resulted in compact plants producing high numbers of long-lasting flowers in a range of flower colours and shapes. *Streptocarpus* species are desirable due to their interesting flower morphology and different growth type. Thus, a combination of both would be interesting in terms of generating novelty, which is of special importance in ornamental plants.

Interspecific hybridisation is a source of variation and an important tool to create new cultivars in ornamental breeding. Interspecific hybrids between *Saintpaulia ionantha* and species of the subgenus *Streptocarpella* have not successfully been produced by conventional sexual hybridisation (Afkhami-Sarvestani 2010). However, the development of biotechnological procedures in recent decades has generated new possibilities for plant breeding, and somatic hybridisation has been used to overcome the barriers of sexual incompatibility (e.g. in cyclamen: Prange et al. 2012). To produce new ornamentals, the objectives of this study were (i) to identify an appropriate starting material for protoplast isolation from two *Streptocarpus* species, (ii) to develop a protocol for protoplast culture in these species, (iii) to establish a protocol for somatic hybridisation between *Saintpaulia ionantha* and

Streptocarpus species, and (iv) to identify somatic hybrids by flow cytometry and molecular markers.

Materials and Methods

Plant material

Experiments were conducted using two varieties of *Saintpaulia ionantha:* 'Claudia' with violet flowers and 'De Eeuwige Lente' with white flowers, and two species of the genus *Streptocarpus*, subgenus *Streptocarpella: S. saxorum* × *S. stomandrus* and *S. caulescens*. Plants of these four genotypes were cultivated in the experimental greenhouse and in vitro shoot cultures were established as described previously (Grunewaldt 1977; Afkhami-Sarvestani et al. 2006).

Tissue culture media

Media for the source plant materials for protoplast isolation. Axenic plantlets were maintained on a modified MS medium (Murashige and Skoog 1962) with an NH_4NO_3 concentration that was reduced to 2 mM and 30 g l^{-1} sucrose, 1 mg l^{-1} thiamine-HCl, 100 mg l^{-1} myo-inositol and 8 g l^{-1} agar (Serva, Heidelberg, Germany, Nr. 11396). The medium for *Saintpaulia ionantha* was plant growth regulator-free (Grunewaldt 1977), whereas for *Streptocarpus* species, 0.1 mg l^{-1} NAA (naphthalene acetic acid) and 2 mg l^{-1} BAP (6-benzyladenine) were added (Afkhami-Sarvestani et al. 2006). In addition, the medium for *Streptocarpus* was prepared with and without agar. Shoot regeneration media were supplemented with 0.8 mg l^{-1} IAA and 0.4 mg l^{-1} BAP for *Saintpaulia ionantha* (Grunewaldt 1977) and with 1 mg l^{-1} NAA and 2 mg l^{-1} BAP for *Streptocarpus* (Afkhami-Sarvestani et al. 2006). All media were adjusted to pH 5.8 with 0.1 M KOH or HCl and autoclaved at 121 °C under 1.1 kg cm^{-2} of pressure for 20 minutes.

Media for protoplast culture. The composition of the protoplast culture media (PCM), callus induction media (CIM I and II), shoot regeneration medium (SRM) and shoot elongation medium (SEM) for *Saintpaulia ionantha* were prepared according to Winkelmann and Grunewaldt (1992). For the protoplast culture of *Streptocarpus* and the fusion products, variations in $CaCl_2$ concentration in the protoplast culture media (PCM: 1,200 mg l^{-1} $CaCl_2$ and 2 mg l^{-1} NAA, CIM I: 1,800 mg l^{-1} $CaCl_2$ and 1 mg l^{-1} NAA) were tested. The pH of the protoplast culture media was adjusted to 5.6, and they were filter sterilised. To solidify the CIM II, SRM and SEM, 3 % agarose (Biozym LE Agarose, Nr. 840004) was mixed with the liquid media (2.5 parts medium with 1 part agarose).

For shoot regeneration from protoplast-derived calluses of *Streptocarpus*, media B5 (Gamborg et al. 1968) and BPM (Winkelmann and Grunewaldt 1992), with various growth regulators, were compared (Table 1).

The enzyme solution for *Saintpaulia ionantha* was prepared according to Winkelmann and Grunewaldt (1992) to obtain a final concentration of 1.0 % cellulase "Onozuka" R-10 and 0.5 % macerozyme R-10 (Duchefa, Haarlem, The Netherlands). The final concentration of cellulase and macerozyme for protoplast isolation for *Streptocarpella* was adjusted to 1.0 % each.

Protoplast isolation

Protoplast isolation of *Saintpaulia ionantha* was carried out according to Winkelmann and Grunewaldt (1992) as

Table 1. The combinations of plant growth regulators and silver nitrate that were tested for shoot induction from protoplast-derived calluses of *Streptocarpus* [mg l^{-1}].

Variant	IAA	NAA	BAP	2iP	TDZ	Zeatin	Kinetin	Meta-topolin	Adenine sulphate	Silver nitrate
A	0.5		4.0							
B		0.05			1.0					
C	0.5			4.0				2.0		
D	0.5			4.0						
E		0.50		2.0					7.0	
F_1		5.00	5.0							3.0
F_2			3.0			1.0				
G	1.0					4.0				
H	1.0						4.0			
I_1		0.50	0.5							
I_2		0.01	5.0							

Note: According to Akasaka-Kennedy et al. (2005), the calluses of variant F_1 were transferred to medium F_2 after one week, and the calluses of I_1 were cultured on medium I_2 after two weeks.

follows: leaf blade explants were cultured on regeneration medium and incubated at 26 °C in the dark for six to eight weeks. The regenerated white small shoots and shoot primordia on the explants were used as the source material for protoplast isolation (Fig. 1a). The shootlets (0.2 to 0.4 g) were chopped, incubated for 1–2 h in 5 ml of preplasmolysis solution (0.3 M sorbitol, 0.1 M glycine and 0.05 M $CaCl_2$), then incubated for 15 h in 5 ml of enzyme solution in the dark at 26 °C followed by 1 h on a rotary shaker (30 rpm). The obtained suspension was sieved through a nylon sieve (100 μm) to remove undigested tissue. For further purification, the protoplast suspension was mixed with 5 ml of 20 % sucrose overlaid with 1.5 ml of a modified washing solution (60 mM sucrose, 300 mM $CaCl_2$, 252 mM KCl, 2 mM NH_4NO_3 and 1 mM KH_2PO_4 (Girmen et al. 1991)). After centrifugation at 100 × g for 6 min, the protoplasts were collected from the interface and transferred to a new centrifuge tube containing 5 ml of the washing solution. Protoplasts were pelleted by centrifugation at 60 × g for 6 min.

For the protoplast isolation from *Streptocarpus*, the following source materials were tested:

1. After surface sterilisation with 1.5 % sodium hypochlorite, the leaves, stems and petals of greenhouse-grown plants treated in the enzyme solution with and without vacuum. Vacuum-filtration was applied for 5–8 min until no air bubbles rose from the plant tissue.
2. The regenerated shootlets on the leaf blade explants that had been cultured at 26 °C in either a 16-h photoperiod (28 μmol m^{-2} s^{-1}) or in continuous darkness for six to eight weeks (Fig. 1b).
3. The leaves and stems of in vitro plantlets, which had been grown on solidified medium at 26 °C in either a 16 h photoperiod by 8 μmol m^{-2} s^{-1} or in continuous darkness. The leaves were treated with or without vacuum.
4. The leaves and stems of in vitro plantlets cultured in liquid media (Fig. 1c): 2–3 plantlets were placed in 250 ml flasks containing 10–25 ml of liquid medium so that the tips of the shoots were not submersed. The culture was incubated on a rotary shaker at 40 rpm at 24 °C in darkness. Fresh medium was added weekly according to the growth of the shoots.

The viability of the protoplasts was determined using FDA (fluorescein diacetate) according to Widholm (1972) by mixing 0.1 ml of protoplast suspension with a drop of 0.25 % FDA solution in DMSO (Serva, Heidelberg, Germany). After 10 min, approximately 500 protoplasts were evaluated using a Zeiss IM35 inverted microscope at 100x magnification with the filter combination of BP 450-490, FT 510, and LP 520.

Protoplast culture and plant regeneration

For embedding, the protoplasts were re-suspended in 0.5 M mannitol and mixed with the same volume of Na-alginate (2.1 % in 0.44 M mannitol) to obtain a final density of 1.5×10^5 protoplasts per ml. The mixture was pipetted in aliquots on $CaCl_2$-agar (0.02 M $CaCl_2$ in 0.5 M mannitol). After one hour, solidified alginate films containing the embedded protoplasts were transferred to a 3.5 cm diameter plastic Petri dish with 1.5 ml of PCM and incubated at 26 °C in the dark. After 5 days, the liquid PCM was renewed. The PCM was replaced with CIM I 14 days after plating, which was again renewed after another 5 days. Five days later, the osmolarity was further reduced using CIM II. This medium was renewed every week. When microcalluses had reached a size of approximately 1 mm in diameter, the alginate films were dissolved in a solution consisting of 0.2 M citric acid monohydrate and 0.2 M sucrose (pH 5.6). The microcalluses were subcultured on solidified CIM II every two weeks. Approxima-

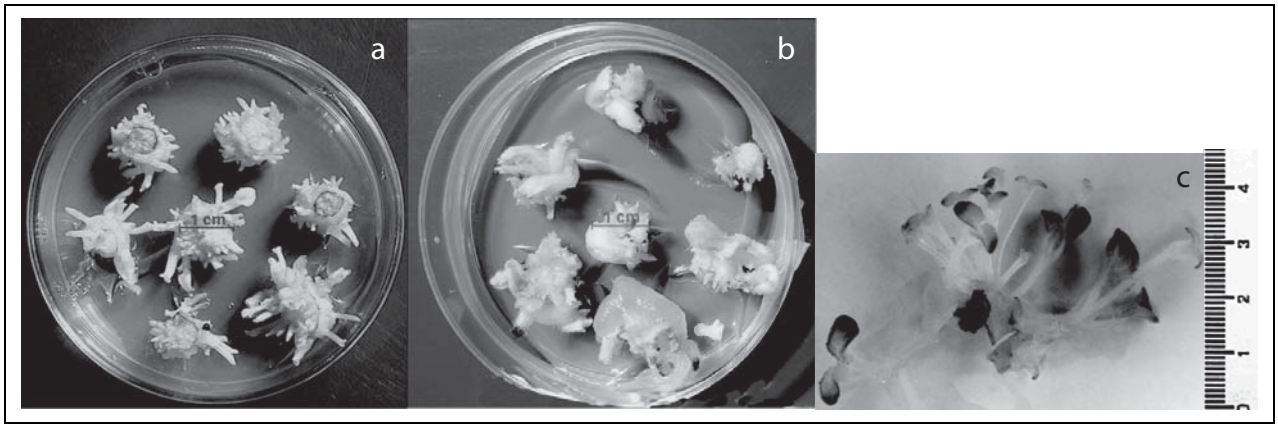


Fig. 1. Starting material for protoplast isolation: regenerated shoots on leaf blade explants of *Saintpaulia ionantha* (a) and *Streptocarpus caulescens* (b) and plantlet of *Streptocarpus caulescens* cultured in liquid medium (c).

tely two months later, the calluses were transferred to shoot regeneration medium (SRM) and the media listed in Table 1.

Protoplast fusion

After the first washing, leaf protoplasts of the two *Streptocarpus* species and protoplasts that were isolated from the young shootlets of two *Saintpaulia ionantha* genotypes were suspended at a density of 1×10^6 ml^{-1} in a solution (modified after KAO and MICHAYLUK 1975) containing 0.4 M glucose, 3.5 mM $CaCl_2$ and 0.7 mM KH_2PO_4. Two PEG (polyethylene glycol) solutions were used for fusion: solution 1 (0.2 M glucose, 2 M PEG 6,000 (3 g PEG/10 g H_2O), 8 mM $CaCl_2$ and 0.7 mM KH_2PO_4) and solution 2 (additionally containing 50 mM HEPES (2-(4-(2-hydroxyethyl)-1-piperazinyl) ethanesulphonic acid) to stabilise the pH at 5.6). For fusion, 0.5 ml of PEG solution was pipetted into a 12 ml centrifuge tube. The tube was agitated to distribute the PEG evenly along the inner walls. Thereafter, the same volume (0.5 ml) of protoplast suspension was gently added drop wise onto the film of the PEG solution. After the protoplasts had settled (approximately 20 min), 1 ml of BPM (pH 9.5) was added dropwise into the tube. After 1 min, 3 ml of BPM (pH 5.6) were added to the mixture and the tube was gently agitated to resuspend the protoplasts evenly in the solution. Ten minutes later, the purification was started using two methods: (i) 5 ml of modified washing solution according to GIRMEN et al. (1991) was pipetted into a tube followed by centrifugation at 60 × g for 6 min. This procedure was repeated with 10 ml of washing solution. (ii) The fusion products were mixed with 5 ml of 20 % sucrose solution, and the following steps were carried out as described above for the protoplast isolation.

To stain the nuclei in the protoplasts (YE and EARLE 1991), the pellet was resuspended in 0.5 M mannitol with 0.5 ml of staining solution (5 parts 0.5 M sorbitol, 10 mM $CaCl_2$, and 5 mM MES, 3 parts 95 % ethanol and 2 parts ethidium bromide (freshly prepared from a 10 mg ml^{-1} stock solution at 98 % in water)). After 30 min, the protoplasts were centrifuged at 5,000 rpm, the pellet was resuspended in staining solution, and approximately 500 protoplasts were evaluated for their number of nuclei using a Zeiss IM35 inverted microscope at 100x magnification with the filter combination of BP 450-490, FT 510, and LP 520.

Identification of fusion products

For the flow cytometric estimation of the DNA content, 0.5 cm² of leaf blade tissue from in vitro plantlets or 10–30 mg of calluses that were derived from fusion experiments were chopped on ice with a sharp razor blade in 400 µl of solution A from the high resolution kit (Partec, Münster, Germany). The samples were then filtered through a 50 µm sieve and stained with 1,600 µl of staining solution B (containing DAPI (4′,6-diamidino-2-phenylindol)). After 2 min, the relative fluorescence intensity of at least 2,000 particles was measured using a flow cytometer (CA II, partec) and analysed by the DPAC software (Partec).

For RAPD (random amplified polymorphic DNA) analyses, genomic DNA was extracted from fresh in vitro leaf tissue or calluses using the DNeasy Plant Mini Kit (Qiagen). A random primer from Kit A (Carl Roth, Karlsruhe, Germany: A09: 5'GGGTAACGCC3') was used. PCR was performed in a 20 µl reaction solution containing 2.5 µl of Williams buffer (10 x) (WILLIAMS et al. 1990), 2 µl of dNTPs (1 mM), 2 µl of primer (5 pM), 4 µl of Taq DNA polymerase (0.25U µl^{-1}, Axon Labortechnik, Kaiserslautern, Germany), 9.5 µl of H_2O and 5 µl of total DNA (4 ng µl^{-1}). Amplification was carried out in a T3 Thermocycler (Biometra, Goettingen, Germany) at 94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 92 °C for 1 min, 35 °C for 1 min, and 72 °C for 2 min, ending with 72 °C for 10 min. The amplified fragments were electrophoretically separated at 70 V for 1 h in 1.5 % agarose gels (Biozym LE agarose, Hessisch Oldendorf, Germany) containing 1.5 µg ml^{-1} ethidium bromide in TAE buffer (SAMBROOK et al. 1989) and photographed under UV light (320 nm).

Results

Protoplast isolation and culture

Protoplasts of *Saintpaulia ionantha* were isolated, cultured and regenerated successfully according to WINKELMANN and GRUNEWALDT (1992). In contrast, it was not possible to directly apply this protocol to *Streptocarpus*. The shootlets that were regenerated on the leaf explants of *Streptocarpus* were hyper-hydrous and sometimes turned brown (Fig. 1b compared to Fig. 1a). The stems and leaves of greenhouse-grown plants, despite removing the lower epidermis and using a vacuum, released only a few protoplasts.

Shoot cultures that had been grown under light on solid medium gave rise to low protoplast yields of 0.05–0.15 × 10^5 g^{-1} fresh mash (FM) for leaves, with only slight improvements seen using vacuum filtration of the enzyme solution (Table 2). Higher yields, especially for *S. caulescens*, were achieved if stems served as the source material; however, this material was available in limited quantities and showed enormous variation among different isolations (Table 2).

In further experiments, different light intensities and liquid medium were compared for in vitro shoot cultures. These experiments showed that the protoplast yield varied not only with the genotype but depended highly on the type of source material and light intensity used during pre-culture. Reducing the amount of light during the culture of the source material increased the protoplast yield but simultaneously led to browning of the starting material tissue (Fig. 1b) and to much lower viabilities (Table 3).

Table 2. Protoplast yields (x 10^5 g^{-1} FM) from the leaves and stems of in vitro grown shoots of the two *Streptocarpus* species (cultured under higher light intensity, with and without vacuum application).

	No. of isolations	Leaves		Stems
		without vacuum	with vacuum	
S. saxorum × *S. stomandrus*	8	0.05 ± 0.02	0.10 ± 0.10	0.14 ± 0.13
S. caulescens	8	0.09 ± 0.04	0.15 ± 0.10	1.77 ± 1.10

Table 3. Yield (x 10^5 g^{-1} FM) and viability of the protoplasts that were isolated from the leaves and stems of in vitro grown shoots and young regenerated shootlets of two *Streptocarpus* species depending on the culture conditions. The data are given as the means and standard deviations of 8 replicates from 2 independent isolations.

	Regenerated shootlets on leaf explants		Plantlet culture							
			on solid medium						in liquid medium	
	28 µmol m^{-2} s^{-1}	dark	28 µmol m^{-2} s^{-1}		8 µmol m^{-2} s^{-1}		dark		dark	
	shoot	shoot	leaf	stem	leaf	stem	leaf	stem	leaf	stem
					Yield					
S. saxorum × *S. stomandrus*	0.93 ± 0.40	1.70 ± 0.72	0.98 ± 0.62	1.92 ± 0.83	1.27 ± 0.25	3.25 ± 2.60	1.14 ± 0.41	3.16 ± 1.23	3.44 ± 1.76	0.92 ± 0.17
S. caulescens	1.52 ± 0.61	5.88 ± 1.06	0.79 ± 0.45	1.35 ± 0.48	1.61 ± 0.92	3.18 ± 1.44	1.85 ± 0.40	2.45 ± 0.82	3.63 ± 1.14	0.79 ± 0.54
					Viability					
S. saxorum × *S. stomandrus*	74 %	57 %	75 %	77 %	36 %	42 %	35 %	44 %	96 %	71 %
S. caulescens	80 %	58 %	79 %	80 %	28 %	25 %	26 %	25 %	80 %	83 %

Only the leaves and stems of plantlets that had been grown in liquid media in the dark did not show browning (Fig. 1c) and released a large number of protoplasts (Table 3, Fig. 2a). The protoplasts varied in diameter between 35 and 52 µm, which was very similar to *Saintpaulia ionantha* protoplasts (30–35 µm).

The first cell division was observed 9–11 days after protoplast isolation, and most cells divided more or less symmetrically (Fig. 2b). To optimise the PCM for *Streptocarpus*, the concentration of calcium and auxin were varied. While doubling the NAA concentration led to an increase in cell division for *Str. saxorum* × *Str. stomandrus* from 0.7 % to 2.3 % and for *Str. caulescens* from 1.1 % to 2.2 %, doubling the calcium concentration enhanced the division frequencies only by 0.2 % and 0.5 %, respectively (data not shown). Thus, the PCM used for all further *Streptocarpus* protoplast cultures contained 2.0 mg l^{-1} NAA and 1,200 mg l^{-1} $CaCl_2$.

Cell divisions were only observed in protoplasts that were derived from regenerated shootlets (under light), stems of in vitro shoots grown in low light conditions and leaves of shoots grown in liquid media in the dark (Table 4). However, the division frequencies after 14 days were quite low (approximately 2–4 %, Table 4). Interestingly, sustained cell division, microcallus and callus formation was only recorded for protoplasts that were isolated from leaves of shoots that were grown in liquid media and in darkness (Table 4, Fig. 2c, d).

When the calluses had reached 1 mm in diameter, they were separated from the alginate films and transferred to solidified CIM II. Overall, 238 calluses of *S. saxorum* × *S. stomandrus* and 345 calluses of *S. caulescens* were obtained from 6 isolations each. At first they were white, spherical and very solid in structure, and shortly after exposure to light they turned green (Fig. 2d). At a size of 3–5 mm, the calluses were transferred to various shoot regeneration media (Table 1). On these media, containing diverse combinations of plant growth regulators, only root formation was recorded at different frequencies, but shoot regeneration was not observed.

Protoplast fusion

When treated with the PEG solution, the protoplasts immediately agglutinated to form clumps of two or more protoplasts (Fig. 3a). Nuclei stained with ethidium bromide

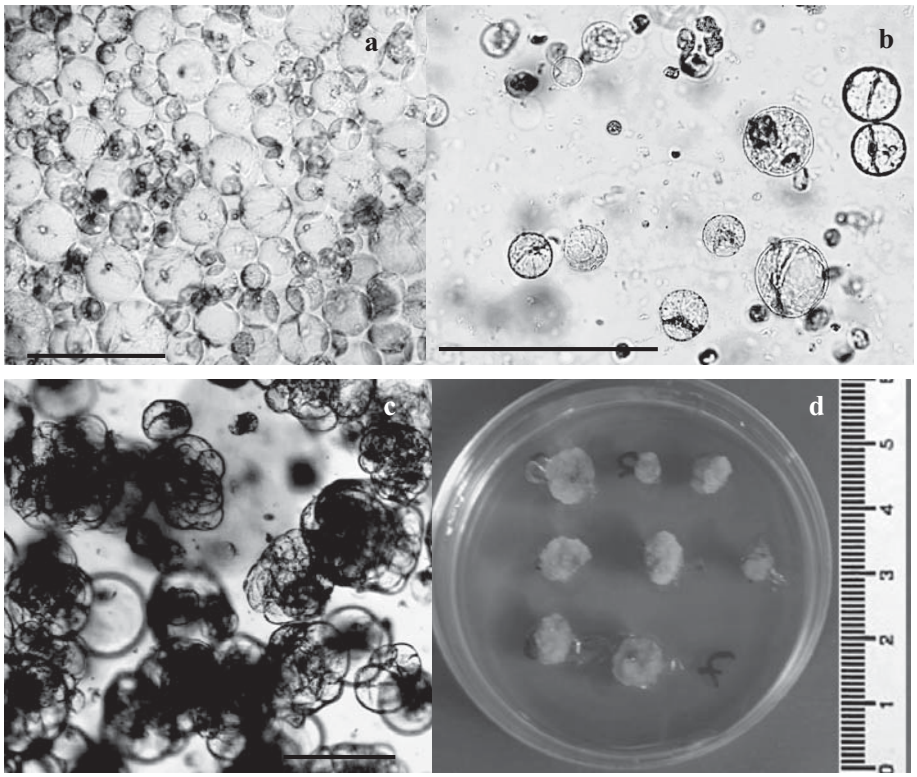


Fig. 2. Protoplasts released from leaves of *Streptocarpus caulescens* after pre-culture of the in vitro shoots in liquid medium in darkness (a), first cell divisions 11 d after protoplast isolation in *S. saxorum* × *S. stomandrus* (b), microcallus formation in *Streptocarpus caulescens* 3 weeks after isolation (c) and calluses of *Streptocarpus caulescens* 3 months after isolation (d). Bars represent 200 µm.

Table 4. Division frequencies [%] of the cells that were derived from the protoplasts of *Streptocarpus* species after 14/28 days, depending on the starting material used. The data are given as the means and standard deviations of 8 replicates from 2 independent isolations.

	Regenerated shootlets on leaf explants	*In vitro* plantlets cultured	
		on solid medium	in liquid medium
	dark	low light	dark
	shoot	stem	leaf
	After 14 d		
S. saxorum × *S. stomandrus*	2.3 ± 1.1	1.8 ± 1.5	3.9 ± 1.2
S. caulescens	2.5 ± 1.5	–	4.5 ± 2.5
	After 28 d		
S. saxorum × *S. stomandrus*	3.5 ± 1.7	1.8 ± 0.6	11.5 ± 3.1
S. caulescens	3.3 ± 2.1	–	13.2 ± 0.9

showed bright red fluorescence, making them easy to see and count (Fig. 3b). Unfortunately, this method does not differentiate between homo- and heterofusion products.

The stability of the pH in the fusion solution was achieved due to the addition of 50 mM HEPES. During autoclaving, the pH in the unbuffered PEG solution dropped to 3.9, whereas HEPES stabilised the pH at 5.7 after autoclaving.

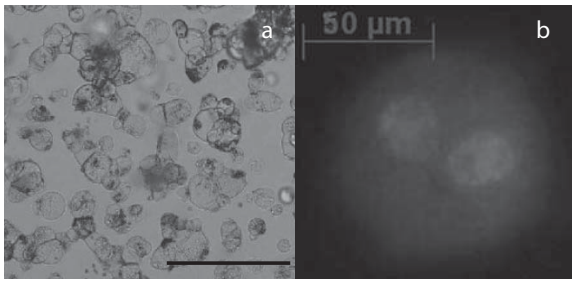


Fig. 3. Agglutination of protoplasts 1 min. after application of PEG solution 1 (a), bar represents 200 µm. Binuclear protoplast after fusion stained by ethidium bromide (b).

The effect of the two different pH values (5.6 and 9.5) in the PEG solutions was compared in fusion experiments involving protoplasts of *Streptocarpus caulescens* and *Saintpaulia ionantha*. The microscopic observation showed that at a pH of 5.6, significantly higher fusion frequencies (16 % binuclear protoplasts) were obtained than were observed in the unbuffered control (7.2 %, Table 5). Even higher frequencies (23.3 %) were observed after treatment with the high pH PEG solution, but a pronounced decline in viability was also noticed.

High portions of protoplasts were destroyed by the fusion treatment that destabilises membranes and involves mechanical stress. The broken protoplasts most likely released toxic compounds resulting in decreased development and cell divisions after fusion in the first experiments. Only further purification by floating the intact protoplasts on a 20 % sucrose solution led to cell division. Furthermore, this purification treatment resulted in a slight increase in viability and a pronounced rise in the frequency of binuclear protoplasts (Table 6). Further development, cell division and the development of microcalluses and calluses were similar to that observed in the protoplast culture of *Streptocarpus* (as described above).

The optimised fusion protocol was applied in all combinations of the two *Streptocarpus* species and the two *Saintpaulia ionantha* genotypes to achieve intergeneric somatic hybrids (Table 7). High variation was observed in terms of numbers of Petri dishes in which further development was noticed. While in the fusion combination d (*Streptocarpus caulescens* × *Saintpaulia ionantha* 'De Eeuwige Lente') calluses formed in all three replications, the other three combinations did lead to cell divisions in

Table 5. Effect of the pH during PEG treatment on the viability and fusion frequency (determined as percentage of binuclear protoplasts) in fusion experiments between *Saintpaulia ionantha* and *Streptocarpus caulescens*. The viability and percentage of binuclear protoplasts before PEG treatments were referred to as control. The data are given as the means and standard deviations of 2 replicates.

	Control	pH: 5.6 (PEG solution 1)	pH: 9.5 (PEG solution 2)
Viability	81.0 ± 16.5	63.0 ± 21.7	48.0 ± 23.3
Binuclear	7.2 ± 2.6	16.0 ± 4.4	23.3 ± 8.9

Table 6. The viability and binuclear protoplast formation (%) before and after the fusion treatments and after further purification through flotation on a 20 % sucrose solution. The data are given as the means and standard deviations of 2 replicates.

	Before fusion	After fusion	After purification
Viability	89.0 ± 4.1	64.0 ± 13.0	68.0 ± 5.4
Binuclear	6.8 ± 2.2	15.3 ± 6.5	35.7 ± 4.1

Table 7. Overview of the results of the protoplast fusion experiments between two *Streptocarpus* species and two varieties of *Saintpaulia ionantha* in 3 replications.

	Replication 1							Replication 2							Replication 3						
Fusion experiment	No. of independent fusions	No. of Petri dishes	No. of Petri dishes with cell divisions	% cell division after 14 d[1]	% cell division after 28 d[1]	No. of microcalluses	No. of calluses	No. of independent fusions	No. of Petri dishes	No. of Petri dishes with cell divisions	% cell division after 14 d[1]	% cell division after 28 d[1]	No. of microcalluses	No. of calluses	No. of independent fusions	No. of Petri dishes	No. of Petri dishes with cell divisions	% cell division after 14 d[1]	% cell division after 28 d[1]	No. of microcalluses	No. of calluses
a	2	6	4	0.7	2.5	3	3	3	5	4	1.2	3.5	12	10	1	3	0	0.0	0.0	0	0
b	4	8	8	1.2	5.5	18	18	2	3	0	0.0	0.0	0	0	5	11	0	0.0	0.0	0	0
c	3	7	4	0.0	0.0	0	0	6	14	5	2.2	10.7	12	12	4	10	0	0.0	0.0	0	0
d	4	7	5	3.4	20.2	400	342	3	6	6	4.0	12.0	105	92	2	4	4	2.9	9.8	9	9

a: *Streptocarpus saxorum* × *S. stomandrus* x *Saintpaulia ionantha* 'Claudia'

b: *Streptocarpus caulescens* × *Saintpaulia ionantha* 'Claudia',

c: *Streptocarpus saxorum* × *S. stomandrus* x *Saintpaulia ionantha* 'De Eeuwige Lente'

d: *Streptocarpus caulescens* × *Saintpaulia ionantha* 'De Eeuwige Lente'

[1] mean of Petri dishes with cell divisions

much lower frequencies (Table 7). In total, 486 calluses were obtained from fusions, the majority of which (443 calluses) were derived from the fusion of *Streptocarpus caulescens* × *Saintpaulia ionantha* 'De Eeuwige Lente' (Table 7). However, all attempts to induce shoot formation on these calluses involving different plant growth regulator combinations failed.

Hybrid callus verification

The first intention was to identify fusion calluses by flow cytometry. Therefore, the relative DNA content of leaf material from both genera was measured. Because of the similarity of genome sizes between *Streptocarpus* and *Saintpaulia* species (Fig. 4a and 4b), the identification of somatic hybrids by flow cytometry was not applicable. However, the selection of calluses with a doubled genome size compared to the parental species was possible (Fig. 4c). Therefore, the calluses were screened by flow cytometry and those with a peak position between 100 and 140 were estimated to be tetraploid. Depending on the fusion combination, between 33 and 56 % of the analysed calluses fell into this category, between 23 and 34 % were of higher ploidy, and fewer than 32 % had obviously developed from unfused protoplasts (Fig. 5).

The hybridity of the 30 tetraploid calluses was tested by RAPD analysis. Fig. 6 shows that one callus derived from the fusion of *Streptocarpus caulescens* and *Saintpaulia ionantha* 'De Eeuwige Lente' was confirmed to be a somatic hybrid callus expressing markers from both parents (callus no. 12). For 27 of the remaining calluses, the band patterns did not clearly show fragments of the *Saintpaulia ionantha* parent and more closely resembled the *Streptocarpus* parent, whereas only for one callus the *Saintpaulia* band patterns were detected. Additionally, the fact that no shoots could be regenerated most likely indicates that *Streptocarpus* protoplasts better tolerated the fusion treatment. However, the molecular marker analysis gave evidence that two calluses expressed bands of both parental species and additional new bands. Thus, the fusion of protoplasts between the two genera was possible and hybrid calluses could be obtained.

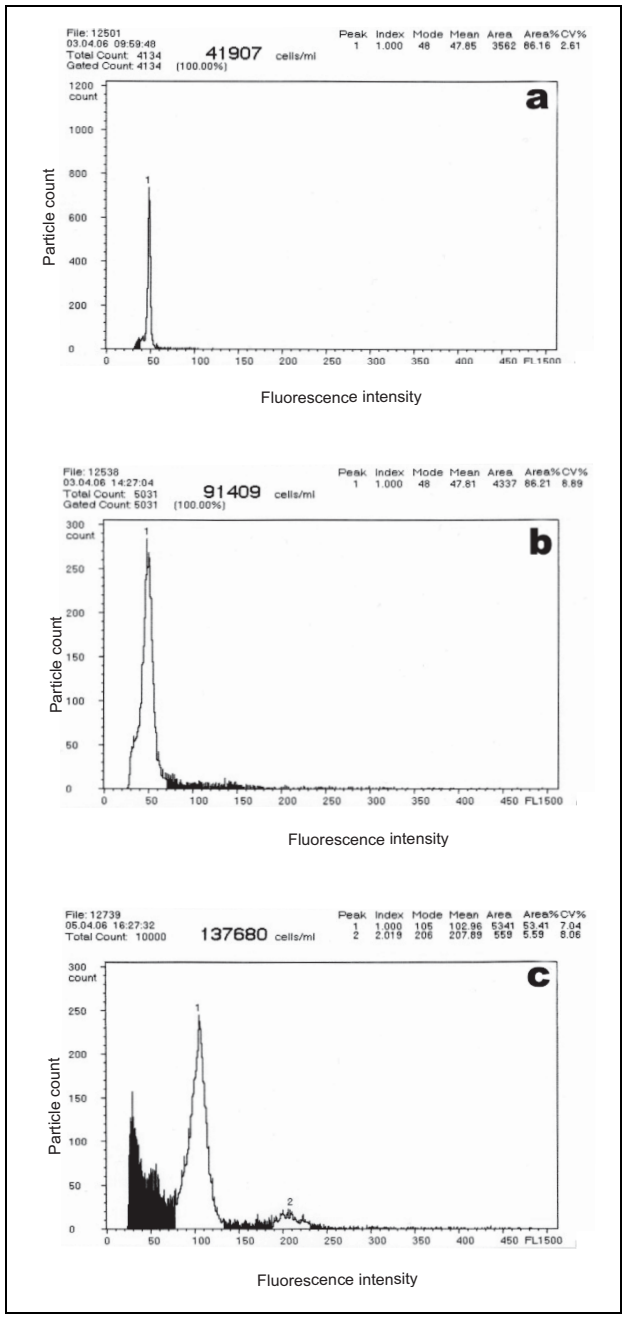


Fig. 4. Flow cytometric analysis of in vitro leaf material of *Santpaulia ionantha* (a), *Streptocarpus caulescens* (b) and a tetraploid callus derived from a protoplast fusion experiment of both (c).

Discussion

High numbers of viable protoplasts can be isolated from in vitro shoot cultures grown in liquid medium in the dark.

A large number of viable protoplasts are essential for a successful protoplast fusion approach. The isolation, culture and plant regeneration of protoplasts from *Saintpaulia ionantha* were successfully established according to WINKELMANN and GRUNEWALDT (1992, 1995a, b), but this method could not be directly adopted for *Streptocarpus* species (Fig. 1, Table 4 and 5). Therefore, suitable plant material for protoplast isolation had to be initially identified. Protoplasts in appropriate yields were released from the leaves of in vitro shoot cultures that had been incubated in liquid media in the dark (Table 4).

Leaf tissue has often been a source of plant protoplasts because it allows for the isolation of a large number of relatively uniform cells without destroying the plants (BHOJWANI and RAZDAN 1996), but in some species, for instance *Limonium*, yields were insufficient (KUNITAKE and

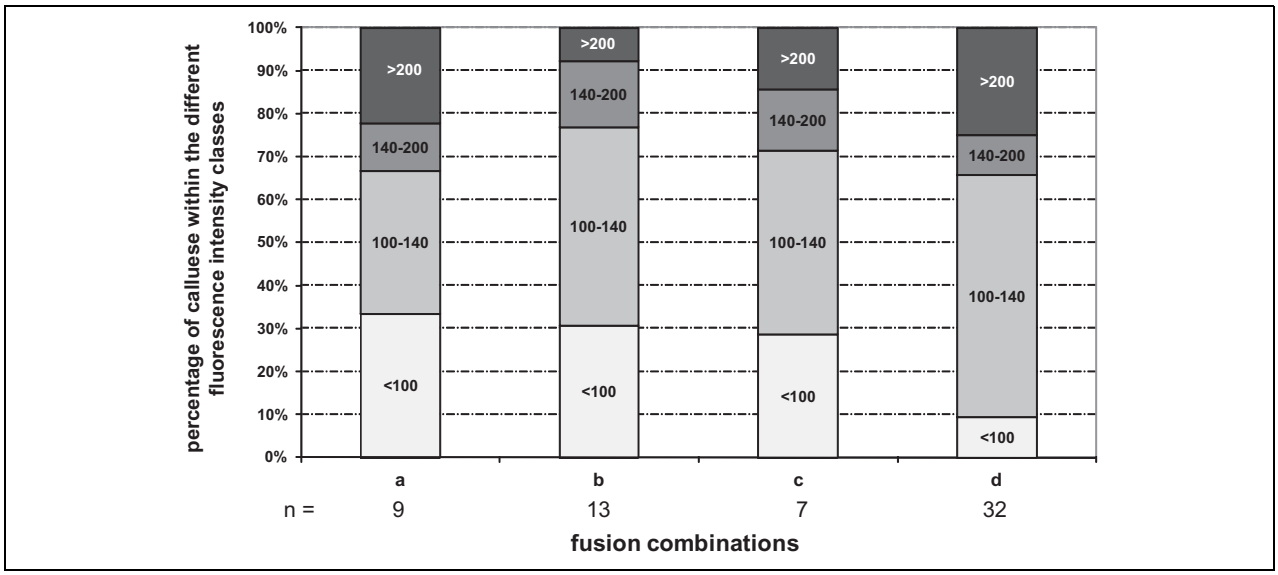


Fig. 5. Percentages of fusion-derived calluses showing specific fluorescence intensities in flow cytometric analyses. The calluses showing only one major peak were classified. The number of classified calluses is given as n below the fusion combinations. a: *Streptocarpus saxorum* × *S. stomandrus* × *Saintpaulia ionantha* 'Claudia'; b: *Streptocarpus caulescens* × *Saintpaulia ionantha* 'Claudia'; c: *Streptocarpus saxorum* × *S. stomandrus* × *Saintpaulia ionantha* 'De Eeuwige Lente'; d: *Streptocarpus caulescens* x *Saintpaulia ionantha* 'De Eeuwige Lente'.

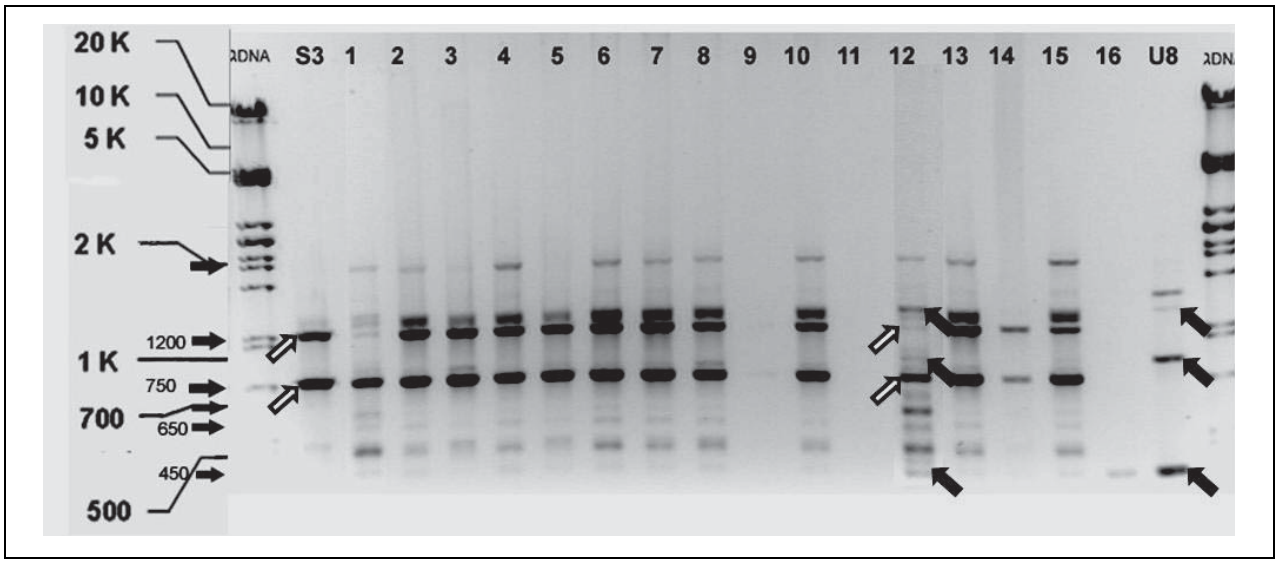


Fig. 6. RAPD analysis of 15 fusion-derived calluses and parental species (primer A15) S3 = *Streptocarpus caulescens* (white arrows indicate prominent fragments of this parent), U8 = *Saintpaulia ionantha* 'De Eeuwige Lente' (black arrows indicate specific fragments of this parent).

Mii 1990). To facilitate the penetration of the enzyme solution into the intercellular space of the leaves, the lower epidermis of leaves from cereals and *Citrus* were peeled, and a vacuum was applied (Scott et al. 1978; Grosser 1994). Although these methods increased the yield of protoplasts in *Streptocarpus* as well (Table 2), they did not result in sufficient protoplast numbers.

The reduction of the light intensity during the culture of the starting material was successful in increasing the protoplast yield in *Dianthus* (Nakano and Mii 1992), *Citrus* (Grosser 1994) and *Saintpaulia* (Winkelmann and Grunewaldt 1992, 1995a, b; Hoshino et al. 1995). Accordingly, protoplast yields were also enhanced in this study, but tissue browning and protoplast viability simultane-

ously decreased, and cell divisions stopped early in the culture. The deleterious effects of low light intensities were counterbalanced only if the shoots were incubated in liquid medium.

Streptocarpus protoplast culture was successful, but only until callus formation.

Using suitable source material and optimised media, callus formation was reproducibly obtained in both of the *Streptocarpus* species tested. These calluses grew vigorously, but after transfer to a range of different plant growth regulator combinations (including BAP, 2,4-D (2,4-dichlorophenoxyacetic acid), kinetin, 2iP (2iP – 6-(γ,γ-dimethylallylamino)purine), TDZ (thidiazuron), zeatin, meta-topolin, NAA, IAA (indole acetic acid) and adenine sulphate) and silver nitrate to block ethylene perception, only root formation could be observed. Additionally, different light intensities or different nutrient compositions did not result in shoot regeneration (data not shown). The lack of success in shoot induction could either be due to endogenous plant hormone concentrations, such as high endogenous auxin levels or low sensitivity to the applied cytokinins, or to the starting material used for protoplast isolation. In many studies, shoot induction from mesophyll protoplasts has been described as being difficult or impossible, most likely because of the high differentiation of the mesophyll cells (Evans and Cocking 1975). Additionally, in *Saintpaulia ionantha*, plant regeneration from protoplasts depended strongly on the source material (young regenerated shootlets) (Winkelmann and Grunewaldt 1992). Hoshino et al. (1995) regenerated plants from protoplasts of *Saintpaulia ionantha* that were isolated from suspension cultures. However, this protocol was not successful when applied to *Streptocarpus* in our hands (data not shown). Other studies made use of embryogenic suspension cultures as starting material (e.g., in *Cyclamen* (Winkelmann et al. 2006; Prange et al. 2010) or rose (Schum et al. 2001; Kim et al. 2003)). It may be assumed that, especially in recalcitrant species, the source material for protoplast isolation is essential.

Development of PEG-mediated protoplast fusion between Saintpaulia ionantha and Streptocarpus species

For protoplast fusion, it is necessary that plants can be regenerated from protoplasts of at least one fusion partner. Therefore, experiments aiming at the identification of a method for somatic hybridisation between *Saintpaulia* and *Streptocarpus* species were carried out, although our attempts to regenerate shoots on the protoplast-derived calluses of *Streptocarpus* were not successful. At first, 30 % PEG applied for 20 min was identified to be optimal among the diverse PEG concentrations tested (data not shown). Two major influencing factors reported in this study were the pH during the fusion treatment and the purification after the fusion. The high alkaline pH (9.5) during fusion caused severe damage to the protoplasts and the loss of protoplasts due to bursting. Therefore, for the subsequent fusion experiments, a pH of 5.6 was chosen. The toxic effects of high pH values on the viability of protoplasts have also been described for *Vicia* (Kao and Michayluk 1975) and *Pisum* (De Filippis et al. 2000).

Although high pH values are known to support the fusion process, as indicated by the higher percentages of binuclear protoplasts in this study (Table 5), complete fusions were also observed in the lower pH treatment.

In addition to removing broken protoplasts, the sucrose flotation treatment resulted in a higher percentage of binuclear protoplasts (36 %). This might be explained by additional fusion events during centrifugation. Kao and Michayluk (1975) and Zimmermann and Scheurich (1981) found that the change in osmolarity of protoplasts during washing and centrifugation could also cause higher fusion rates.

Future experiments should evaluate the possibility of staining the parental protoplasts with different dyes because we were not able to visually distinguish between them. Recently, this staining approach allowed the clear detection of heterofusion frequencies in *Cyclamen* (Prange et al. 2012). The frequencies given in this study for binuclear protoplasts of greater than 30 % seem to be very high and presumably contain many homofusion events. Another approach would be the use of complementary metabolic inhibitors for both parental protoplasts, as shown for iodoacetate and rhodamine 6-G in *Nicotiana* (Böttcher et al. 1989), however, in our hands their effects were not reproducible.

When the calluses that were derived from fusion experiments were analysed by flow cytometry, a high portion turned out to be polyploid with many calluses being tetraploid (Fig. 5). Their frequency was even higher than that of the binuclear protoplasts, indicating the possibility that tetraploid cells were more vigorous than diploid ones. These tetraploid calluses could have originated from either the desired heterofusion events, but also from homofusions and from spontaneous fusion as well as chromosome doubling during callus culture as previously reported for *Saintpaulia ionantha* (Winkelmann and Grunewaldt 1995b).

Hybrid verification by DNA markers detected two intergeneric somatic hybrid callus. Thereby, it was shown that somatic hybridisation between these two genera is possible in principle. As discussed above, more investigations to achieve shoot regeneration are required in the future. Although, for example, in *Citrus*, the regeneration ability of one partner is sufficient (Grosser and Gmitter 2011), it might be necessary to first solve the problem of shoot regeneration from protoplast-derived calluses in *Streptocarpus* before hybrid plants are produced.

In conclusion, this is the first study to report protoplast isolation and culture from the genus *Streptocarpus*, the culture of plantlets in liquid media as a source material,

and the somatic hybridisation between *Streptocarpus* and *Saintpaulia*.

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