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Spontaneous polyploidisation of interspecific and intersectional *Pelargonium* hybrids during embryo rescue

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Summary

Modern *Pelargonium crispum* hybrids (section *Pelargonium*) show low genetic and phenotypic variation due to the domestication effect. Species of the sections *Cortusina*, *Ligularia*, and *Pelargonium* are potential breeding partners at the diploid level ($2n = 2x = 22$). Five *P.* × *crispum* cultivars were used as seed parents and pollinated with one genotype of *P. grandiflorum* (section *Pelargonium*) and three genotypes of *P. fulgidum* (section *Ligularia*). In both combinations, embryo rescue was necessary. Embryos were rescued and cultured on Murashige & Skoog medium supplemented with phytohormones. After callus and adventitious shoot regeneration 15 viable interspecific hybrids were obtained from crossbreeding with *P. grandiflorum* and 11 intersectional hybrids from crossings with *P. fulgidum*, respectively. The hybrids were cultivated in the greenhouse until flowering. Their hybrid character was evident due to the intermediate morphological traits. Molecular investigations using dp-RAPD analysis confirmed this. Within the F_1 population *P.* × *crispum* with *P. grandiflorum* three hybrids and after crossing with *P. fulgidum* one hybrid possessed larger flowers and fully developed anthers, respectively. Their ploidy level was confirmed as tetraploid using flow cytometry. Therefore, a spontaneous polyploidisation occurred during *in vitro* regeneration. The tetraploid F_1 hybrids are fertile and could be used for further breeding.

Key words: Genome doubling, flow cytometry, *Ligularia*, *Pelargonium crispum*, *Pelargonium fulgidum*, *Pelargonium grandiflorum*, ploidy level, somaclonal variation

Introduction

Angel or pansy pelargoniums (*P. crispum* (P.J. Bergius) L'Hér. hybrids) (section *Pelargonium* (DC.) Harv.) are popular ornamental plants already for centuries. Their percentage on the production of bedding plants increases continuously. In the 1920s, the documented breeding of Angel pelargoniums started in England done by Langley-Smith (Brawner, 2003). However, it is believed that the interspecific hybridisation with *P. crispum* began much earlier soon after the import of different *Pelargonium* species to Europe during the 17th century (Brawner, 2003).

Modern *P. crispum* hybrids, which should be referred as *P.* × *crispum* (Olbricht, 2013), show low genetic (Plaschil et al., 2017) and phenotypic variation due to crossings within a very narrow gene pool during domestication (Brawner, 2003). Increase of genetic variability by crosses with wild species could overcome this genetic bottleneck (Plaschil et al., 2012, 2015; Olbricht, 2013).

The genus *Pelargonium* L'Hér. with its about 280 species (Bakker et al., 1998, 2004, 2005; Weng et al., 2012) in 16 sections (Bakker et al., 1999a, b, 2000, 2004, 2005; Röschenbleck et al., 2014) comprises species of different sections such as *Cortusina* (DC.) Harv., *Ligularia* (Sweet) Harv. and *Pelargonium*, which are potential breeding partners for *P.* × *crispum* at the diploid level ($2n = 2x = 22$). A study of genetic distances of several species of the working collection at the Julius Kühn Institute, Quedlinburg provides additional valuable information on potential crossing partners (Plaschil et al., 2017).

Natural diploid hybrids of the section *Pelargonium* are known, e.g. *P. cucullatum* (L.) L'Hér. × *P. betulinum* (L.) L'Hér. and *P. scabrum* (L.) L'Hér. × different diploid species (Albers and van der Walt, 1984; van der Walt et al., 1990; Bakker et al., 2004, 2005). In addition, experimental hybrids were created several times. Thus *P. grandiflorum* (Andr.) Willd. was used as pollen parent for crosses with *P. fruticosum* (Cav.) Willd, *P. crispum* and *P. cucullatum* and as seed parent for crosses with *P. cucullatum*, *P. glutinosum* (Jacq.) L'Hér., and *P. cordifolium* (Cav.) Curtis (Horn, 1994). Yu (1985) successfully hybridised the species *P. crispum* with *P. grandiflorum* and Olbricht (2013) *P. grandiflorum* with *P.* × *crispum*, respectively.

Pelargonium fulgidum (L.) L'Hér. (section *Ligularia*), with its very attractive bright red flower colour, was one of the most popular species used for crossings in the early 19th century (Albers et al., 1992). Together with *P.* × *domesticum* L.H. Bailey, *P. fulgidum* is reputed to be an ancestor of the cultivar group Uniques, but the pedigrees are not well documented (Brawner, 2003). To a small extent Uniques are propagated for satisfying the wishes of few enthusiasts in Europe and America. However, the extraordinary flower colour of *P. fulgidum* is still missing in modern *P.* × *crispum* assortments.

Based on these facts one genotype of *P. grandiflorum* (section *Pelargonium*) and three genotypes of *P. fulgidum* were chosen as pollen donors for crossings with *P.* × *crispum* cultivars to enhance the genetic variability.

Materials and methods

Plant material

Five *P.* × *crispum* cultivars were used as seed parents (Tab. 1). *Pelargonium grandiflorum* and three genotypes of *P. fulgidum* (11, 123, 48) were the pollen donors. All genotypes, maintained as a clone with at least three plants, were cultivated under greenhouse conditions at the Julius Kühn Institute. The internal standards for flow cytometric investigations, radish (*Raphanus sativus* L.), tomato (*Solanum lycopersicum* L.) 'Stupické' (Doležel et al., 1992) and cauliflower (*Brassica oleracea* L. subsp. *capitata* convar. *botrytis* var. *botrytis* L.) 'Korso' (Plaschil et al., 2020) were cultivated *in vitro* on Murashige and Skoog (MS) medium (1962) with 1.07 µM 1-naphtalene acetic acid at 25 °C at 16 h photoperiod.

Cross Combinations and Embryo Rescue

Each *P.* × *crispum* cultivar was crossed with each pollen donor (Tab. 1). For that, flower buds of the seed parents were emasculated

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Tab. 1: Pelargonium genotypes used for crossing

Seed parents (short names)	Pollen donors*
P. × *crispum* 'Piccola™ Harlekin' ('Harlekin')	*P. grandiflorum* (DEU648PELAR038)
P. × *crispum* 'Piccola™ Merlot' ('Merlot')	*P. fulgidum* 11 (DEU648PELAR034)
P. × *crispum* 'Piccola™ Soft Pink' ('Soft Pink')	*P. fulgidum* 123 (DEU648PELAR036)
P. × *crispum* 'Piccola™ Pink Picotee' ('Pink Picotee')	*P. fulgidum* 48 (DEU648PELAR035)
P. × crispum 'Angeleyes® Randy' ('Randy')	

* https://www.bundessortenamt.de/apps6/genbank_zierpfl/public/de

and isolated. The stigmas, when ripen, were pollinated with pollen from at least one anther of the pollen donor. Reciprocal crosses were not carried out, because of the low flower number of *P. grandiflorum* and *P. fulgidum*.

Fourteen to thirty-one days after pollination unripe fruits were collected and surface sterilised in a sodium hypochlorite solution (3% active chlorine) (Carl Roth, Karlsruhe, Germany) followed by threefold rinsing with autoclaved distilled water. Embryos were dissected under sterile conditions and cultivated on MS medium (Duchefa, Haarlem, The Netherlands) supplemented with 2.28 µM zeatin (Duchefa), 1.14 µM indole-3-acetic acid (Duchefa), and 1.44 mM gibberellic acid 3 (Duchefa) in petri dishes (ø 6 cm) at 24 °C in the dark. One week later, a light exposure of 16 h (108 $\mu mol/m^{-2}s^{-1}$, provided by cool white fluorescent lamps, Philips F17T8/TL741 Alto, 17 W, U.S.A.) alternated by 8 h darkness per day was applied. When the cultivated embryos formed adventitious shoots, the shoots were separated, multiplied, and rooted on MS without phytohormones. Rooted microplants were transferred into Fruhstorfer® sowing and cutting substrate and later potted in a mixture of Stender® substrate C420 and sand (1:1). Firstly, they were cultivated in a climate chamber. After hardening cultivation took place in the greenhouse until flowering.

Molecular hybrid identification

Total DNA was isolated using the protocol of Porebski et al. (1997) with some modifications: 100 mg fresh leaf material were disrupted in a 1.5 mL tube with 400 µL preheated extraction buffer by a stainless steel grinding ball using a mixer mill MM300 (Retsch, Haan, Germany) two times for 5 minutes with a frequency of 30 s^{-1}. Finally, the DNA was resuspended in 50 µL TE buffer. Concentration and purity of the DNA were spectrophotometrically determined to adjust all samples to 10 ng μL^{-1} DNA. RAPD analysis was performed according to Williams et al. (1990) but using a mixture of two decamer primers (dpRAPD, Carl Roth, Karlsruhe, Germany) (Budahn et al., 2009) to get more and smaller DNA fragments. The amplification products were separated on 1% agarose gels and stained with ethidium bromide solution. Size determination was realised by comparison to a 100 bp DNA ladder (Fischer Scientific, Carlsbad, CA, USA).

Flow cytometric and statistical analysis

Three to seven biological replications of each genotype were analysed with one or two of the three internal standards. Radish (2C = 1.11 pg; Doležel et al., 1992), tomato 'Stupické' (2C = 1.96 pg; Doležel et al., 1992) and cauliflower 'Korso' (2C = 1.31 pg; Plaschil et al., 2020) were used as internal standards to estimate the DNA content and ploidy level of the investigated genotypes. Sample preparation, calculation of the 2C values and data analysis were performed as described by Plaschil et al. (2020).

Results

Fertilisation, embryo rescue and plant regeneration

Regarding the pollen donor *P. grandiflorum* 77 flowers of the five *P.* × *crispum* cultivars, which were pollinated, resulted in 45 fruits and 88 embryos. Between four flowers ('Harlekin') and twenty-seven flowers ('Soft Pink') were pollinated per cultivar. Within the crossing group *P.* × *crispum* × *P. fulgidum*, 319 flowers were pollinated, 173 fruits were harvested and 227 embryos rescued. Per cross combination between five flowers ('Harlekin' × *P. fulgidum* 11) and 41 flowers ('Merlot' × *P. fulgidum* 123) were pollinated. The detailed results of all twenty cross combinations are shown in Tab. 2 and 3, respectively.

The *in vivo* embryo development was insufficient so that the hybrid embryos started to abort about 14 days after pollination. At that time, we start to take the embryos *in vitro*. After a successful fertilisation, 1 - 3 embryos could be dissected per fruit. The hybrid *Pelargonium* embryos underwent *in vitro* a callus stage with soft yellow callus followed by formation of adventitious shoots eight to twelve weeks later. On average, 17% of rescued embryos from the crossings *P.* × *crispum* × *P. grandiflorum* and 5% from *P.* × *crispum* × *P. fulgidum* resulted in viable plants, respectively.

In both crossing groups the percentage of fruit set per pollinated flowers was nearly similar (58.4% versus 54.2%). Nevertheless the cross combinations have a strong influence on the embryo development (Tab. 2, 3). A difference in the number of developed embryos per fruits was noticed. Whereas in the cross combination *P.* × *crispum* × *P. grandiflorum* predominantly more than one embryo could be dissected this was rarely the case in the crossing group with *P. fulgidum*. The further development of the embryos was sig-

Tab. 2: Regeneration success of hybrids after embryo rescue from crossings *P.* × *crispum* × *P. grandiflorum*

Seed parents	pollinated flowers	Number of fruits	rescued embryos	Explants with adventitious shoots		In vitro microplants		Greenhouse plants	
				total	%[1]	total	%[1]	total	%[1]
'Harlekin'	4	3	7	4	57	4	57	4	57
'Merlot'	23	6	9	0	0	0	0	0	0
'Soft Pink'	27	22	42	14	33	12	29	8	19
'Pink Picotee'	10	6	9	3	33	2	22	1	11
'Randy'	13	8	21	9	43	5	24	2	10
Crossing group Σ	**77**	**45**	**88**	**30**	**34**	**23**	**26**	**15**	**17**

[1]Percentage relating to the number of cultivated embryos, rounded to whole numbers

Tab. 3: Regeneration success of hybrids after embryo rescue from crossings *P.* × *crispum* × *P. fulgidum* 11, 123 and 48

Crossings	pollinated flowers	Number of fruits	rescued embryos	Explants with adventitious shoots		In vitro microplants		Greenhouse plants	
				total	(%)[1]	total	(%)[1]	total	(%)[1]
'Harlekin' × 11	6	6	6	0	0	0	0	0	0
'Merlot' × 11	40	18	28	2	7	2	7	0	0
'Soft Pink' × 11	23	10	6	0	0	0	0	0	0
'Pink Picotee' × 11	17	11	5	0	0	0	0	0	0
'Randy × 11	28	22	31	2	6	1	3	1	3
Pollen donor 11 Σ	**114**	**67**	**76**	**4**	**5**	**3**	**4**	**1**	**1**
'Harlekin' × 123	5	3	1	0	0	0	0	0	0
'Merlot' × 123	41	15	24	7	29	7	29	5	21
'Soft Pink' × 123	16	10	10	0	0	0	0	0	0
'Pink Picotee' × 123	15	7	8	0	0	0	0	0	0
'Randy' × 123	32	25	33	2	6	2	6	2	6
Pollen donor 123 Σ	**109**	**62**	**80**	**9**	**11**	**9**	**11**	**7**	**9**
'Harlekin' × 48	6	4	5	1	20	1	20	0	0
'Merlot' × 48	36	10	25	5	20	4	16	1	4
'Soft Pink' × 48	19	11	20	0	0	0	0	0	0
'Pink Picotee' × 48	9	3	1	0	0	0	0	0	0
'Randy' × 48	20	16	20	5	25	5	25	2	10
Pollen donor 48 Σ	**96**	**44**	**71**	**11**	**16**	**10**	**14**	**3**	**4**
Crossing group Σ	**319**	**173**	**227**	**24**	**11**	**22**	**10**	**11**	**5**

[1]Percentage relating to the number of cultivated embryos, rounded to whole numbers

nificantly better in the cross combination with *P. grandiflorum*.
Concerning the fertilisation and regeneration success, there were also big differences according to the cross combination. In the crossing group *P.* × *crispum* × *P. grandiflorum*, 'Harlekin' and 'Soft Pink' were the best seed parents. Seventy-five percent of the pollinated flowers of 'Harlekin' and 81.5% of 'Soft Pink' showed fruits and cultivable embryos giving four and eight viable plants in the greenhouse. On the other hand, with 'Merlot', despite the fruit set, embryo development during *in vitro* culture was completely absent.
In the crossing group with *P. fulgidum* embryos with seed parents 'Soft Pink' and 'Pink Picotee' did not grow further. In the combination 'Harlekin' × 48 one rescued embryo could be cultivated to a microplant but died later on. Only five of fifteen combinations exclusively with the seed parents 'Merlot' and 'Randy' resulted in viable F_1 hybrids. Regarding the three pollen donors, the highest number of plants originated from *P. fulgidum* 123 (seven F_1 hybrids what corresponds to 9.6% of pollinated flowers with 'Merlot' and 'Randy') followed by *P. fulgidum* 48 (three F_1 hybrids, 5.4% of the pollinated flowers) and *P. fulgidum* 11 (one F_1 hybrid, 1.5% of the pollinated flowers).

Fig. 1: Flower and leaf of *P.* × *crispum* 'Soft Pink', the hybrids SG2-3.1 and SG2-3.2 and *P. grandiflorum* (from left to right).

Fig. 2: Flower and leaf of *P.* × *crispum* 'Merlot', the hybrid MF4-2 and *P. fulgidum* 123 (from left to right).

Hybrid identification and characters

For all regenerated hybrids at least three clone plants were cultivated in the greenhouse until flowering. Their hybrid character was evident because of the intermediate morphological traits in flower, leaf, and habit (Fig. 1 and 2). F_1 hybrids of *P.* × *crispum* × *P. grandiflorum* had single rose flowers of intermediate size with fully developed anthers. They always possessed darker marks at the upper petals like *P. grandiflorum* and in some cases additional marks at the lower petals. The intermediate sized leaves were different lobated, the typical zone from the pollen donor *P. grandiflorum* was lacking (Fig. 1). Longitudinal growth was also intermediate. The plants were clearly

more vigorous than those of *P.* × *crispum*, but with a poorer branching. Three out of eight hybrids from the cross 'Soft Pink' × *P. grandiflorum* showed distinct larger flowers than their siblings had. In the greenhouse, two of these hybrid clones (SG2-3, SG3-1) consisted of plants with medium (SG2-3.1, SG3-1.1) and plants with large sized flowers (SG2-3.2, SG3-1.2) (Fig. 1).

F_1 hybrids of *P.* × *crispum* × *P. fulgidum* inherited the red flower colour from the pollen donor and the marks from the seed parent (Fig. 2). They were male sterile with no or rudimentary anthers. The growth was weaker and plants tended to become succulent. Among the five different F_1 hybrids of 'Merlot' × *P. fulgidum* 123, one genotype possessed larger flowers with fully developed anthers and fruit set after open pollination. In addition, dpRAPD analysis confirmed the hybrid character of all regenerated F_1 plants. Specific DNA bands of the seed and pollen parents were detected in the F_1 plants (Fig. 3 and 4).

Flow cytometry and ploidy levels

Six genotypes of the crossing group 'Soft Pink' × *P. grandiflorum* and five genotypes of *P.* × *crispum* × *P. fulgidum* 123, including the assumed tetraploid plants, were proven together with the seed and pollen parents by flow cytometric analysis. The 2C values, calculated with a corresponding internal standard, are given in Tab. 4.

It was shown, that the assumed tetraploid F_1 hybrids of both crossing groups have nearly a doubled 2C value compared to the diploid hybrids. As expected from different 2C values of the pollen donors of both crossing groups, the mean 2C value of the diploid and tetraploid F_1 hybrids, originating from *P. fulgidum*, was higher than mean 2C value of the diploid and tetraploid F_1 hybrids, originating from *P. grandiflorum*. Overall, in the crossing group *P.* × *crispum* × *P. grandiflorum*, spontaneous polyploidisation occurred with a frequency of about 20% and within the crossings *P.* × *crispum* × *P. fulgidum* of about 9%, respectively.

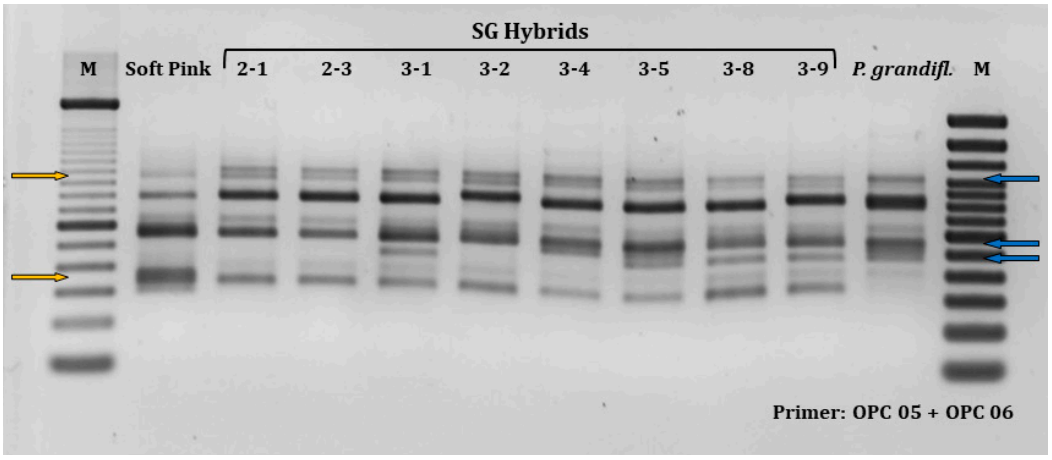

Fig. 3: dpRAPD analysis of eight F_1 hybrids of 'Soft Pink' × *P. grandiflorum* (SG). Specific bands of 'Soft Pink' at about 350 and 960 bp (left arrows) and *P. grandiflorum* at about 490, 590 and 1010 bp (right arrows), respectively (M = two different 100 bp DNA ladders).

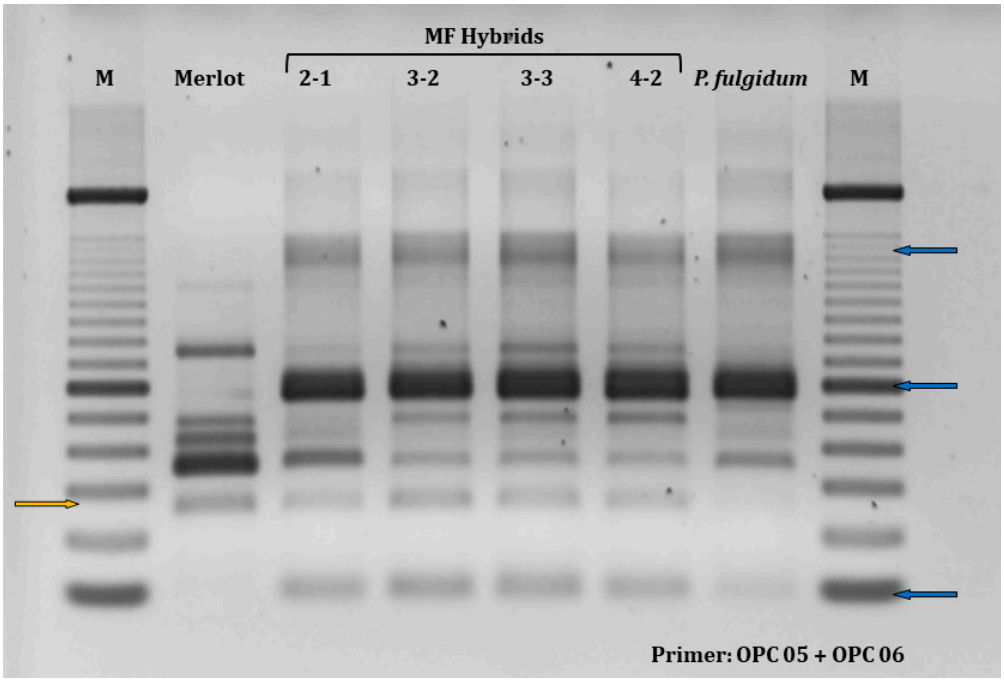

Fig. 4: dpRAPD analysis of four F_1 hybrids of the crossing 'Merlot' × *P. fulgidum* 123 (MF). Specific band of 'Merlot' at about 280 bp (left arrow) and *P. fulgidum* 123 at about 100, 600 and 1350 bp (right arrows), respectively (M = 100 bp DNA ladder).

Tab. 4: Results of the flow cytometric analysis: Mean 2C values of the seed parents, pollen donors and the F1 hybrids of the cross combinations 'Soft Pink' × *P. grandiflorum* and *P.* × *crispum* × *P. fulgidum* 123

Genotype	Internal standard	n	2C value (pg)[1]	SD (±)	Ploidy level
Seed parents					
'Merlot'[#]	'Korso'/'Stupické'	6	1.00[a]	0.05	2*x*
'Soft Pink'[#]	'Korso'/'Stupické'	7	1.01[a]	0.01	2*x*
'Randy'[#]	'Korso'/'Stupické'	7	1.01[a]	0.02	2*x*
Pollen donors and their hybrids					
P. grandiflorum	'Korso'	3	0.99[a]	0.01	2*x*
SG2-3.1*	'Korso'	3	0.98[a]	0.02	2*x*
SG2-3.2*	'Korso'	3	2.07[d]	0.02	4*x*
SG3-1.1*	'Korso'	4	0.97[a]	0.01	2*x*
SG3-1.2*	'Korso'	3	2.09[e]	0.02	4*x*
SG3-4*	'Korso'	3	1.01[a]	0.02	2*x*
SG3-5*	'Korso'	3	2.14[e]	0.02	4*x*
P. fulgidum 11	*Raphanus*	3	1.56[c]	0.09	2*x*
P. fulgidum 123	*Raphanus*	3	1.52[c]	0.09	2*x*
P. fulgidum 48	*Raphanus*	3	1.55[c]	0.05	2*x*
MF2-1**	'Stupické'	4	1.23[b]	0.03	2*x*
MF3-2**	'Stupické'	3	2.59[f]	0.01	4*x*
MF4-2**	'Stupické'	3	1.19[b]	0.01	2*x*
RF4-2**	'Stupické'	3	1.22[b]	0.02	2*x*
RF7-1**	'Stupické'	3	1.19[b]	0.04	2*x*

[#]Data taken from Plaschil et al., 2020; *Cross combination: 'Soft Pink' × *P. grandiflorum* (SG); ** Seed parents: M = 'Merlot', R = 'Randy' and pollen donor F = *P. fulgidum* 123; n = number of biological replications analysed per genotype; SD = standard deviation; 1 different letters indicate significant differences, Tukey's b-test, $\alpha = 5\%$

Discussion

In the genus *Pelargonium*, only intra- and intersubgeneric crossings between genotypes of the same basic chromosome number and ploidy level give a viable, fertile progeny (Yu, 1985; Horn, 1994). The knowledge of phylogeny, genetic distance, variability, ploidy level, and fertility can support plant breeding and interspecific crosses. Although after polyploidisation of *P.* × *crispum* the variability was already widened by crossings with *P.* × *domesticum* at the tetraploid ploidy level (Plaschil et al., 2012, 2015), further interspecific and intersectional hybridisation at the diploid level (2n = 2*x* = 22) is desirable. Reciprocal crosses were omitted, because of fewer flowers of the species compared to the cultivars and the not significant effect on successful fertilisation in *Pelargonium* (Yu, 1985).

Yu (1985) reported about successful interspecific crosses using *P. crispum* as seed parent and *P. grandiflorum* as pollen parent. Six F_1 hybrids regenerated without embryo rescue. It is not known, whether these six hybrids were used for further breeding work. Furthermore, Olbricht (2013) succeeded in crossing *P. grandiflorum* with a breeding clone of *P.* × *crispum* and backcrosses with *P.* × *crispum* until the F_3 generation. Intersectional hybridisation in *Pelargonium* was stated several times (Yu, 1985; Horn, 1994). Yu (1985) described viable hybrids between *P. fulgidum* and diploid Regal pelargoniums (*P.* × *domesticum*, (sect. *Pelargonium*) resulting in cultivars (Brawner, 2003) whereas crosses between *P. fulgidum* and *P. rapaceum* (L.) L'Her. (section *Hoarea* (Sweet) De Candolle) were not successful.

Regarding *P.* × *crispum*, the successful intersectional hybridisation with *P. fulgidum* we reported for the first time, because the breeding pedigree of Uniques was not documented (Brawner, 2003). Even though, it is assumed, that the cultivar pac® Angeleyes® 'Orange', which underwent several hybridisation steps, descended from *P. fulgidum*. One parent of pac® Angeleyes® 'Orange' is the cultivar 'Erin' (breeder Kapac, 1997, USA) and 'Erin' itself should be originated from crosses with *P. fulgidum* (Hofmann & Olbricht, pers. comm.; Olbricht, 2013).

Due to insufficient embryo development after hybridisation, embryo rescue was necessary for both crossing groups as described for other ornamentals (van Tuyl et al., 1991; Winkelmann et al., 2010; Aros et al., 2019). The overall plant regeneration was possible on a low scale of about 3-57% of cultivated embryos. As expected, the regeneration of interspecific *P.* × *crispum* × *P. grandiflorum* hybrids was more successful than that of intersectional *P.* × *crispum* × *P. fulgidum* hybrids, because of the lower genetic distance to the seed parent *P.* × *crispum* (Plaschil et al., 2017).

The regeneration could probably be improved by further investigations concerning the optimal time for fruit harvesting. So far, Scemama and Raquin (1990) only succeeded in direct embryo culture of a diploid *P.* × *hortorum* Bailey, when embryos were removed at least 13 days after pollination, whereas they achieved embryo development and germination as early as 5-6 days after pollination using the ovary culture followed by cultivation of the contained embryos with their scarified seed coat. The authors achieved best results with ovary culture 11 days after pollination combined with the embryo culture five to seven days later. In addition, a more subtle adapted mixture of the *in vitro* medium due to the different genotypes may increase the percentage of viable regenerants (Bentvelsen et al., 1990; Denis-Peixoto et al., 1997; Kamlah et al., 2019).

Through embryo rescue we obtained spontaneous tetraploid F_1 hybrids from both, interspecific cross *P.* × *crispum* × *P. grandiflorum* and the intersectional cross *P.* × *crispum* × *P. fulgidum* showing that the *in vitro* stage is important for the embryo development but it is also an appropriate tool to restore the hybrid fertility and an important prerequisite for further breeding.

Chromosomal disturbances up to a whole genome doubling of *in vitro* plant tissues are a common phenomenon. Firstly LARKIN and SCOWCROFT (1981) summarised these changes under the term somaclonal variation. Spontaneous polyploidisation was often reported in *in vitro* haploid techniques such as microspore, anther or ovule culture, respectively (AHMADI and EBRAHIMZADEH, 2020; BOERMAN et al., 2020; YUAN et al., 2015; CAMPION et al., 1995). The spontaneous chromosome doubling is an important factor for the production of homozygous plants because it is a desirable replacement for colchicine treatment. This phenomenon is described in almost all *in vitro* techniques such as long term cultures (ZIAUDDIN and KASHA, 1990), *in vitro* shoot regeneration from leaves (MEYER et al., 2009) or from embryogenic calli (ISHIGAKI et al., 2014). As far as we know, no polyploidisation of *Pelargonium* during *in vitro* embryo rescue has been described, yet.

The influencing factors on somaclonal variation such as exogenous phytohormones or other chemicals in the nutrient medium, the developmental stage of the explants, and the duration of the *in vitro* culture were comprehensively investigated. Nevertheless, questions remain open for better use of the process of spontaneous chromosome doubling. Obviously, the genetics of the cultivated plants plays a role in induction of the chromosome doubling during the tissue culture. The frequency and regularity of spontaneous polyploidisation during embryo rescue of *Pelargonium* needs further explorations. Already LARKIN and SCOWCROFT (1981) mentioned *in vitro Pelargonium* plants as an example for the presence of somaclonal variations after various *in vitro* cultures such as genetic instabilities and changes of the ploidy. CASSELLS et al. (1995, 1997) confirmed these findings with other *Pelargonium* accessions applying molecular methods. It could be assumed, that the tendency to genetic changes during the *in vitro* phase also made the duplication of chromosomes during *Pelargonium* embryo rescue possible.

Considering the morphological traits of the hybrids, the bright red flower colour of *P. fulgidum* inherited in crossings with *P.* × *crispum* is a promising new feature, but the vigour of the F_1 hybrids was reduced. All diploid hybrids were male sterile with further unfavourable morphological traits. Their value for further breeding as seed parent has to be proved. Probably, the fertility must be restored by polyploidisation. Only the fertile tetraploid hybrid is an appropriate crossing partner. In any case, further backcrosses with *P.* × *crispum* are necessary to establish cultivars.

Hybridisation between *P.* × *crispum* and *P. grandiflorum* is supposed to be a promising enhancement of genetic variation in the very narrow gene pool of *P.* × *crispum* cultivars. The primary diploid and tetraploid F_1 hybrids were vigorous, had attractive flowers with marks and fertile anthers. Hence, they could be used for further breeding at two different ploidy levels, e.g. for backcrosses with *P.* × *crispum* or crossings with *P.* × *domesticum*. The hybridisation success could be increased using embryo rescue. The current results encourage the usage of *Pelargonium* species in breeding programs of *P.* × *crispum* combined with the application of embryo rescue.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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