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[1]Albrecht Daniel Thaer-Institut, Humboldt-Universität zu Berlin
[2]Julius Kühn-Institut, Quedlinburg

Hybridization between *Pelargonium acetosum* L'Hér. and *Pelargonium* ×*peltatum*

R. Kamlah[1]*, I. Pinker[1], S. Plaschil[2], K. Olbricht[1]

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Summary

Pelargonium acetosum L'Hér. is a wild species from South Africa with decorative bluish foliage. Only few reports describe crossings between *P. acetosum* and *P. peltatum* L'Hér. (or *P.* ×*peltatum*). Therefore, information about hybridization barriers is limited. In this study, two different genotypes of *Pelargonium acetosum* (*AC1* and *AC2*) were crossed with the diploid *P.* ×*peltatum* 'Tornado Fuchsia' (*PTF*). Embryos and F_1 hybrids from the combination *AC1* × *PTF* were hampered by chlorophyll deficiencies. Embryos and seeds of the combination *AC2* × *PTF* were underdeveloped. The reciprocal combination *PTF* × *AC1* did not show any fruit set. The combination *PTF* × *AC2* resulted in low numbers of seeds, which were normally developed. Hybrids from seeds were only obtained from the combinations *AC1* × *PTF* and *PTF* × *AC2*. Embryo rescue of the combinations *AC1* × *PTF* and *AC2* × *PTF* resulted in few but viable hybrids. Flowers of all hybrids had shrivelled anthers and proved to be sterile. The occurrence of most hybridization barriers varied strongly between the different combinations and depended on both the genotype and the direction of cross-breeding. The bluish leaf colour did not appear among the F_1. To overcome hybrid sterility a polyploidization is suggested.

Keywords: Embryo rescue, hybridization barriers, hybrid variegation, hybrid sterility, incomplete embryo development, interspecific hybridization, wild species introgression

Introduction

In Europe and North America, *Pelargonium* cultivars represent a significant fraction of the bedding plant market. In order to maintain that position, breeders regularly have to come up with novelties. Interspecific hybridization is one of the main approaches in creating new characteristics, and it counteracts a narrowing gene pool in *Pelargonium* breeding programs (Olbricht, 2013). Worldwide about 280 *Pelargonium* species (Albers and van der Walt, 2007) embody valuable genetic resources that have not yet been fully exploited. However, introgression of species is time-consuming and often hampered by reproductive barriers, which depend, amongst other reasons, on the genetical distance between two species. Interspecific cross-combinations are usually more successful if both species belong to the same section of *Pelargonium*. Other factors influencing interspecific crossability include the ploidy level, the basic chromosome number, and the direction of cross-breeding (Horn, 1994). DNA-based phylogenetic analyses (Bakker et al., 2004; Weng et al., 2012; Röschenbleck et al., 2014) support a subdivision of the genus into 16 sections and help breeders to recognize possible candidates for introgression.

The section *Ciconium* (Sweet) Harv. contains the horticulturally important species *P. inquinans* L'Hér., *P. zonale* L'Hér. (both ancestors of *P.* ×*hortorum* Bailey, the 'zonal geranium'), and *P. peltatum* L'Hér. (the main ancestor of 'ivy-leaved' cultivars, usually named *P.* ×*peltatum*). In recent decades, several species of this section have been introgressed into *P.* ×*hortorum*, such as *P. tongaense* Vorster (Esenalieva et al., 2012) and *P. quinquelobatum* Hochst. (Denis-Peixoto et al., 1997; Hondo et al., 2015). *P. peltatum* was formerly placed in the section *Dibrachya* (Sweet) Harv. but has been included in *Ciconium* (Gibby et al., 1990). *P. peltatum* is characterized by a relatively large genetical distance to other species of the same section (James et al., 2004; Weng et al., 2012). In hybridization experiments between various species of section *Ciconium* and *P. peltatum*, both prezygotic and postzygotic barriers have been observed. An inhibited pollen tube growth or a lack of fertilization (when pollen tubes do grow down the style) represent the main observed prezygotic barriers (Coffin and Harney, 1978; Yu, 1985). Known postzygotic barriers include incomplete development of seeds, stunted plant growth, hybrid sterility, chlorophyll deficiencies and hybrid variegation (Coffin and Harney, 1978; Yu, 1985; Horn, 1994). The latter is a consequence of the biparental inheritance of plastids in *Pelargonium*: If only one of the two inherited plastid types shows an incompatibility with the nuclear genome, the segregation of plastids often leads to variegated leaves with chlorophyll-deficient sectors (Metzlaff et al., 1981, 1982; Grieger, 2007; Weihe et al., 2009).

In the case of an incomplete development or the abortion of the embryo, postzygotic disturbances may be overcome by the use of embryo rescue. In respect to the explant material, embryo rescue techniques can be distinguished into ovary, ovule, and embryo culture (Winkelmann et al., 2010). A first study about embryo rescue of *P.* ×*hortorum* was published by Becker-Zens (1983), in which both ovule and embryo culture were performed. Scemama and Raquin (1990) developed a method to circumvent early embryo abortion using a combination of ovary and embryo culture. Bentvelsen et al. (1990) applied embryo culture in various crosses between *P.* ×*peltatum* and other species of the section *Ciconium*. All these studies used phytohormone-free nutrient media and aimed at the germination of the embryo, while other studies applied growth regulators to induce callus and adventitious shoots (Kato and Tokumasu, 1983; Kakihara et al., 2012).

Pelargonium acetosum L'Hér. (sect. *Ciconium*) is a species from South Africa with $2n=2x=18$ chromosomes. It stands out due to its decorative bluish foliage, which is not common among ornamental *Pelargonium* cultivars. Despite its distinct appearance, it is closely related to *P. zonale* (James et al., 2004), and has been successfully introgressed into *P.* ×*hortorum* (Hondo et al., 2014). A hybridization between *P. acetosum* and *P.* ×*peltatum* using embryo rescue is documented by Bentvelsen et al. (1990), resulting in F_1 hybrids but no F_2 or backcross (BC) generation. Yu (1985) described crossings between *P. acetosum* and *P. peltatum* as not resulting in viable hybrids, while Horn (1994) reported viable but sterile hybrids from the combination *P. peltatum* × *P. acetosum*.

The objective of this study was to examine hybridization barriers between *P. acetosum* and *P.* ×*peltatum* and to obtain genotypes with a novel variability for further breeding purposes. The long-term breeding aim is to achieve *P.* ×*peltatum* cultivars with bluish foliage.

* Corresponding author

Materials and methods

Plant material and cultivation

Two different genotypes of *Pelargonium acetosum* (*AC1* and *AC2*) and the diploid cultivar *P.* ×*peltatum* 'Tornado Fuchsia' (*PTF*) were used as crossing parents. *AC1* was ordered from a nursery (Gärtnerei Schoebel, Germany), *AC2* was provided by the Julius Kühn-Institut, Quedlinburg. Both genotypes were received as adult plants and propagated by cuttings. *PTF* had to be grown from seeds (mail-ordered from Mary K's Unique Seeds, USA). The cultivation of the plants took place from 08/2016 until 01/2018 in the greenhouse (Berlin-Dahlem). Plants were potted in a mix of organic substrate (Klasmann-Deilmann 5) with 10% sand and were fertilized with 0.6% Wuxal Super every 14 days. Assimilation lighting was added using sodium vapour lamps (11 h/d in 2016, 13.5 h/d from 01/2017 until 04/2017, 11 h/d until 07/2017). The temperature ranged between 17 and 35 ºC.

Pollen viability test

Pollen of *AC1*, *AC2,* and *PTF* was stained with an aqueous solution of 1% thiazolyl blue tetrazolium bromide (MTT) and 10% sucrose for 30 min at 24 ºC (Norton, 1966; Firmage and Dafni, 2001). From each genotype 3 × 100 pollen grains were evaluated under a transmission light microscope (Olympus IX70-S8F2). The number of pollen grains showing a purple colour was determined.

Pollination

Flowers were emasculated in the bud stage using forceps (ca. three days before anthesis, when petals became visible at the bud tip). Emasculated inflorescences were covered in perforated propylene bags (Crispac, 11 × 25 cm), which were sealed with Tesa Velcro strips. About five days after emasculation, when the stigma lobes had unfolded, anthers of the crossing partner were pressed on the stigma for pollination. Pollinated flowers were bagged as described above. The number of pollinated flowers varied in each experiment (see below).

Observation of embryo development

For quantitative analysis of embryo development, unripe fruits were harvested two weeks after pollination and the number of embryos per pollinated flower was determined. In each performed combination (Tab. 1) 20 flowers were pollinated.

For qualitative examination of embryo development, unripe fruits were harvested two, three, four, and five weeks after pollination (combinations see Tab. 1). In each variant (combination × point of time = 24 variants) at least five pollinations were carried out. Ovules were taken out of the fruit and were dissected under the stereo microscope (Olympus SZ-PT) with the help of forceps and a dissecting needle. Isolated embryos were documented with a connected camera (Olympus UC30) and associated software (Cellsens Entry 1.6).

Seed production and sowing

The combinations performed in order to achieve seeds are listed in Tab. 1. After harvest, seeds were stored in glassine envelopes. Before sowing, ca. 0.5 mm of the seed tip was removed with fingernail clippers. With the scarified tip facing down, seeds were sown into multi-cell trays (cell size: 6 × 3 × 3 cm) filled with moistened sowing substrate (Klasmann-Deilmann 1). Seed trays were placed under a foil tunnel in the greenhouse for 14 days. Four days after sowing, the front end of the tunnel was opened. During the first two weeks after sowing the temperature ranged between 19 and 30 ºC (22.1 ºC mean). Thirteen days after sowing the number of germinated seeds was determined.

Tab. 1: Performed combinations in each experiment

Experiment	Performed combinations
Quantitative analysis of embryo development	*AC1* × *AC1*, *AC1* × *AC2*, *AC1* × *PTF* *AC2* × *AC2*, *AC2* × *AC1*, *AC2* × *PTF* *PTF* × *PTF*, *PTF* × *AC1*, *PTF* × *AC2*
Qualitative analysis of embryo development	*AC1* × *AC1*, *AC1* × *PTF* *AC2* × *AC2*, *AC2* × *PTF* *PTF* × *PTF*, *PTF* × *AC2*
Seed production and sowing	*AC1* × *AC1*, *AC1* × *PTF* *AC2* × *PTF* *PTF* × *PTF*, *PTF* × *AC2*
Embryo rescue	*AC1* × *AC1*, *AC1* × *PTF* *AC2* × *PTF* *PTF* × *PTF*

Three weeks after sowing, seedlings were transplanted into multi-pot trays (5 cm Ø per cavity), which were filled with a mix of organic substrate and perlite (Klasmann-Deilmann Stecklingssubstrat). Twelve weeks after sowing, plants were potted in a mix of organic substrate (Klasmann-Deilmann 5) with 10% sand and cultivated as described (see plant material and cultivation).

Embryo rescue

Fruits from selected combinations (Tab. 1) were harvested two weeks after pollination. They were surface sterilized in 50 ml disinfection solution (3% calcium hypochlorite and one drop of Tween20) for 20 min and then rinsed three times in sterile deionized water. Under sterile conditions, embryos were excised from fruits using a stereo microscope, forceps, and a dissecting needle. Nineteen embryos of *AC1* × *AC1*, 21 embryos of *AC1* × *PTF,* and 20 embryos each of *AC2* × *PTF* and *PTF* × *PTF* were placed in glass tubes (10 × 2.5 × 2.5), which were filled with 10 ml of solid embryo-rescue-medium (ER-medium, Tab. 2). This medium was supplemented with 1 mg L^{-1} (5.71 µM) indole-3-acetic acid (IAA) and 1 mg L^{-1} (4.4 µM) 6-benzylaminopurine (BAP). For the first two weeks, embryos were cultivated in the dark at 22-24 ºC and subsequently transferred to 10 µmol $m^{-2}s^{-1}$ photosynthetic active radiation (PAR).

Five weeks later (eight weeks later in case of *AC2* × *PTF*), the developed callus was divided into smaller portions and then cultivated in glass tubes (10 × 2.5 × 2.5 cm) each filled with 5 ml phytohormone-free active-charcoal-I-medium (AC-I-medium, Tab. 2). The glass tubes were placed at 22-24 ºC and 30 µmol $m^{-2}s^{-1}$ PAR. Once adventitious shoots reached at least 1 cm, they were transferred into culture jars (Sigma-Aldrich, 66 × 59 × 59 mm, enclosed with Magenta B-cap) filled with 20 ml AC-II-medium. The culture jars were placed at 22-24 ºC and 30 µmol $m^{-2}s^{-1}$ PAR for 16 h d^{-1}.

Rooted and unrooted shoots that had reached at least 2 cm length were planted into 4 cm pots filled with a mix of organic substrate (Klasmann-Deilmann Stecklingssubstrat) and 1/3 perlite. Plants were sprayed with water and placed under a transparent cover (to keep air humidity above 60%) at 21 ºC and 60 µmol $m^{-2}s^{-1}$ PAR for 16 h d^{-1}. Two weeks later plants were transferred into the greenhouse. Acclimated plants were cultivated as described (see plant material and cultivation).

Characterization of plants

Crossing parents and progeny were morphologically characterized. Hybrid status was determined based on the morphology and colour of flowers and leaves. Anthers of the hybrids were examined under a stereo microscope. To assess female fertility, stigmas of the hybrids were pollinated with pollen from *PTF*.

Tab. 2: Composition of culture media

Culture medium	Macro- and micronutrients	Growth regulators (µM)		Vitamins (mg L^{-1})		Other components (g L^{-1})	
ER	MS	5.71	IAA	2.5	Thiamine-HCl	30	Sucrose
		4.40	BAP	0.2	Pyridoxine-HCl	7	Agar
				0.2	Biotin		
				100.0	myo-Inositol		
AC-I	MS	-		2.5	Thiamine-HCl	30	Sucrose
				0.2	Pyridoxine-HCl	7	Agar
				0.2	Biotin	3	Active charcoal
				100.0	myo-Inositol		
AC-II	MS	-		2.5	Thiamine-HCl	30	Sucrose
				0.2	Pyridoxine-HCl	7	Agar
				0.2	Biotin	20	Active charcoal
				100.0	myo-Inositol		

ER = embryo rescue, AC = active charcoal, MS = MURASHIGE and SKOOG (1962), IAA = indole-3-acetic acid, BAP = 6-benzylaminopurine

Statistics

Statistical analysis was carried out with IBM SPSS 23. Post-hoc comparisons were made using Tukey's Honestly Significant Difference (HSD) test.

Results and discussion

Characterization of the crossing partners

The two genotypes of *Pelargonium acetosum* (*AC1* and *AC2*) were characterized by a matte bluish-green foliage. In contrast to *AC1*, the leaves of *AC2* were more strongly lobed and their bluish colour was more distinct. While the flowers of *AC1* had a light salmon-pink colour, the flowers of *AC2* were first pale yellow and became white one day after anthesis. Both genotypes exhibited a more or less upright growth habit. The cultivar *P.* ×*peltatum* 'Tornado Fuchsia' (*PTF*) was characterized by a trailing growth, glossy green foliage, and single fuchsia flowers. The leaf blade showed a horseshoe-shaped dark zone. Both *AC1* and *PTF* were flowering vigorously during this study and proved to be robust. In contrast, *AC2* showed a high susceptibility to thrips, which hampered the development of flowers and complicated the use of this genotype.

MTT staining (Tab. 3) resulted in a low percentage of stained pollen grains in the case of *AC1* and a high portion of stained pollen in the case of *AC2* and *PTF*. Stained pollen grains were interpreted as able to germinate on a stigma (FIRMAGE and DAFNI, 2001), and the percentage of stained pollen was understood as a measure of fertility. Based on these assumptions, the male fertility of *AC2* and *PTF* was considered relatively high (PLASCHIL et al., 2017). In contrast, *AC1* could only be assessed as partially male-fertile, which makes this genotype less suitable as a pollen parent.

Tab. 3: Pollen viability of the crossing parents according to the percentage of stained pollen grains after thiazolyl blue tetrazolium bromide (MTT) treatment

Genotype	Mean stained pollen grains (%)[1]	Standard deviation (%)
AC1	38^{z}	16.09
AC2	86^{y}	4.58
PTF	88^{y}	4.00

[1]different letters indicate significant differences (Tukey-HSD, n = 3 × 100, α = 5%)

Tab. 4: Influence of the combination on the number of embryos, two weeks after pollination

Combination	Mean embryo number per pollinated flower[1]	Standard deviation	Total embryo number per combination
AC1 × *AC1*	0.95^{zy}	0.83	19
AC1 × *AC2*	1.65^{y}	1.46	33
AC1 × *PTF*	2.00^{y}	1.38	40
AC2 × *AC2*	4.05^{x}	1.10	81
AC2 × *AC1*	0.00	0.00	0
AC2 × *PTF*	3.35^{x}	1.60	67
PTF × *PTF*	5.45^{w}	1.57	109
PTF × *AC1*	0.00	0.00	0
PTF × *AC2*	0.30^{z}	0.66	6

[1]different letters indicate significant differences (Tukey-HSD, n = 20, α = 5%)

Interspecific Hybridization

Embryo development

Fruits from selfings of *AC1* (*AC1* × *AC1*) contained a maximum of two embryos and a mean of 0.95 embryos (Tab. 4), most likely due to the low fertility of this genotype. Two weeks after pollination, embryos were in transition between the torpedo and the cotyledon stage (Fig. 1). Three weeks after pollination, embryos had already reached their maximum size. The interspecific combination *AC1* × *PTF* resulted in a maximum of four and a mean of two embryos per pollinated flower, but differences to *AC1* × *AC1* were not significant (Tab. 4).

Embryos from the combination *AC1* × *PTF* were less green than embryos from the selfings of *AC1* (Fig. 1). These apparent chlorophyll deficiencies are most likely due to an incompatibility between one of the two plastomes and the nuclear genome (METZLAFF et al., 1981, 1982; WEIHE et al., 2009). Embryos of the combination *AC1* × *PTF* often exhibited a slightly irregular morphology. In particular, the cotyledons were often unequally sized (Fig. 1).

Selfings of *AC2* (*AC2* × *AC2*) resulted in three to five embryos and a mean of 4.05 embryos per pollinated flower (Tab. 4). These numbers can be considered normal because usually no more than five ovules are fertilized in a *Pelargonium* flower (YANO et al., 1975). The interspecific combination *AC2* × *PTF* resulted in a mean of 3.35 embryos per flower, which was not significantly lower compared to *AC2* × *AC2* (Tab. 4). Despite their relatively high number, these hybrid embryos developed poorly. Two weeks after pollination the embryos had reached, at most, an early torpedo stadium (Fig. 1) and did not develop much further in the following weeks. This delayed and then arrested embryo development might have been caused by an irregular endosperm formation resulting from an incompatibility between the parental genomes in the endosperm (LAFON-PLACETTE and KÖHLER, 2015). Such postzygotic disturbances represent one of the most significant hybridization barriers, which potentially can be overcome by embryo rescue (KULIGOWSKA et al., 2016).

Combinations using *PTF* as the seed parent contained considerably smaller embryos and seeds than the previously mentioned combinations (Fig. 1 and 2). In 11 of 20 fruits deriving from the selfings of *PTF* (*PTF* × *PTF*), the number of five embryos was exceeded because carpels contained 'twin embryos'. The occurrence of twin embryos in *Pelargonium* was documented by YANO et al. (1975) and KUBBA and TILNEY-BASSETT (1980). No fruit development and no embryos were observed in the combination *PTF* × *AC1* (Tab. 4), whereas the combination *PTF* × *AC2* resulted in four fruits per 20 pollinated flowers containing 1-2 embryos (a mean of 0.3 embryos per pollinated flower). It appears likely that fertilization was hampered by prezygotic hybridization barriers (and in the case of *PTF* × *AC1* additionally by a low pollen viability). Prezygotic barriers are well-known from interspecific crossings using *Pelargonium peltatum* as the seed parent (YU, 1985). However, in order to confirm the occurrence of prezygotic barriers, it would be necessary to monitor the pollen tube growth within the pistil (WINKELMANN et al., 2010). Despite their small number, embryos from the combination *PTF* × *AC2* seemed to develop normally in comparison with *PTF* × *PTF* (Fig. 1).

Seed morphology and germination

While all the seeds resulting from the selfings of *AC1* germinated, the germination percentage of the combination *AC1* × *PTF* was only 44% (Tab. 5). Seeds from this combination were not as round as those from the selfings of *AC1* (Fig. 2). The development of most seedlings was hampered by chlorophyll deficiencies.

Seeds from the combination *AC2* × *PTF* were small and underdeveloped (Fig. 2). This result agrees with YU (1985), who reported incompletely developed seeds deriving from the combination *P. ace-*

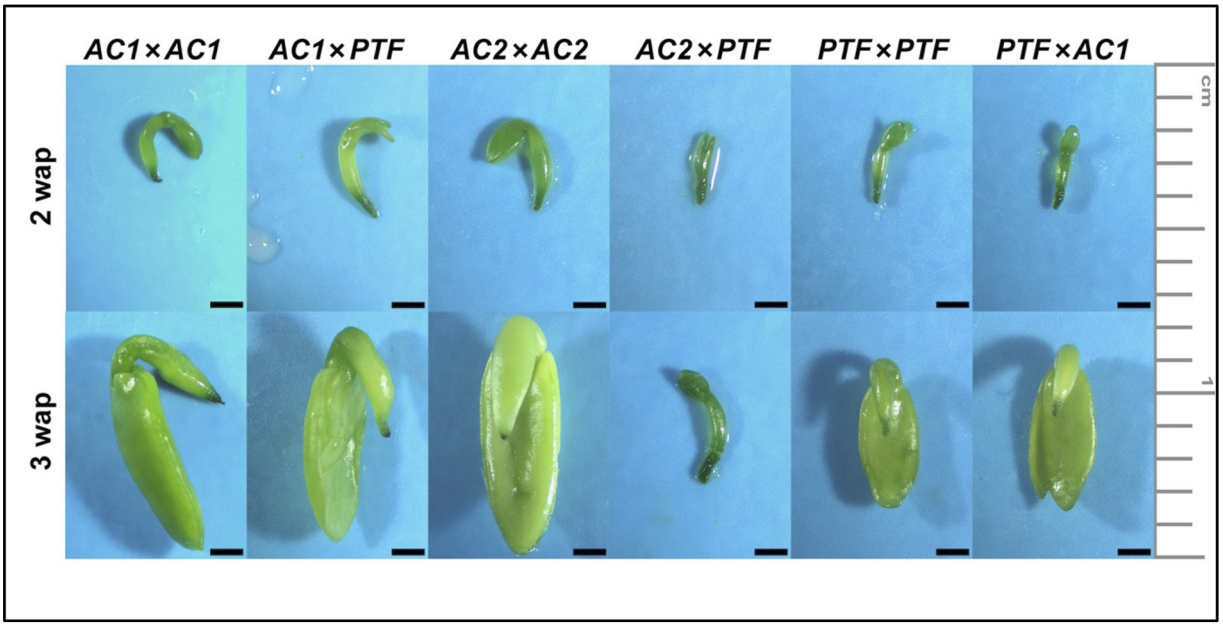

Fig. 1: Morphology of embryos deriving from selfings (control) and interspecific crossings, two and three weeks after pollination (wap), scale bar: 1 mm

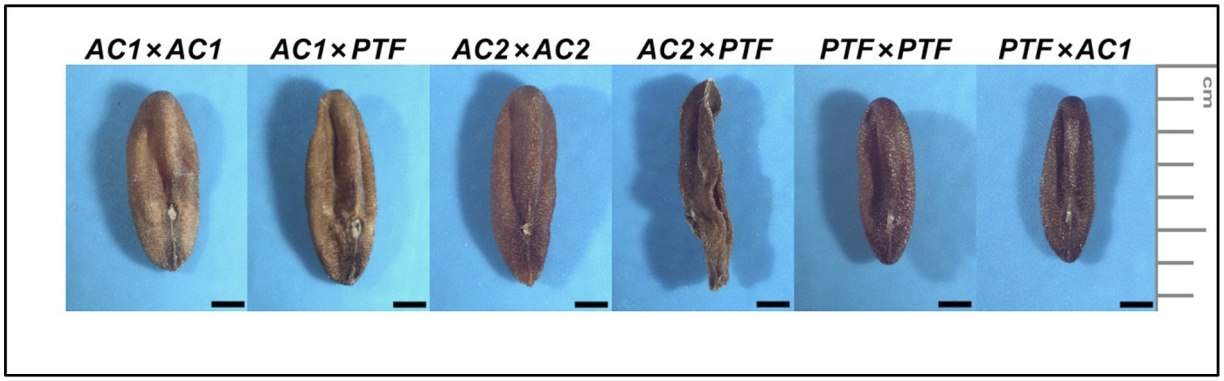

Fig. 2: Morphology of seeds deriving from selfings (control) and interspecific crossings, scale bar: 1 mm

Tab. 5: Influence of the combination on the number of seeds and the germination percentage

Combination	Mean seed number per pollinated flower[1]	Standard deviation	Total seed number per combination	Number of germinated seeds	Germination percentage (%)
AC1 × *AC1*	0.80 ^{zy}	1.06	16	16	100.00
AC1 × *PTF*	1.25 ^{y}	0.64	25	11	44.00
AC2 × *PTF*	2.15 ^{x}	1.35	43	0	0.00
PTF × *PTF*	4.80 ^{w}	1.15	96	73	76.04
PTF × *AC2*	0.30 ^{z}	0.73	6	4	66.67

[1]different letters indicate significant differences (Tukey-HSD, n = 20, α = 5%)

tosum × *P. peltatum*. The small and shrivelled seeds from the combination *AC2* × *PTF* (Fig. 2) did not germinate (Tab. 5). This combination could only be achieved by the application of embryo rescue (see below).

Out of 96 seeds deriving from the selfings of *PTF,* 73 germinated (Tab. 5). The interspecific combination *PTF* × *AC2* resulted in only six seeds per 20 pollinated flowers (a mean of 0.3 seeds per pollinated flower). They appeared normal-shaped (Fig. 2), and four of them germinated (Tab. 5). HORN (1994) reported also a low seed set from the combination *P. peltatum* × P. *acetosum* (a mean of 0.5 seeds per pollinated flower), but in his case the germination percentage was only 36%.

F₁ progeny

Plants from seeds were only obtained from the combinations *AC1* × *PTF* and *PTF* × *AC2*. Except two plants, all hybrids from the combination *AC1* × *PTF* exhibited chlorophyll deficiencies or hybrid variegation, which severely hampered their development (Fig. 3). Consequently, only four plants survived the first six months after sowing. Severe chlorophyll deficiencies leading to a low survival rate in the F_1 also occurred after cross-breeding between *P. acetosum* and *P.* ×*hortorum* (HONDO et al., 2014). However, the four hybrids from the combination *PTF* × *AC2* did not show any chlorophyll deficiencies.

Compared to the plants originating from the selfings of *AC1* and *PTF,* the development of all the interspecific hybrids was at first characterized by considerably short internodes. Then, about 20 weeks after sowing, these hybrids showed elongated internodes and a normal growth habit. However, one hybrid from the combination *PTF* × *AC2* continued growing in a severely stunted manner and did not develop any flowers. Hybrids with such stunted growth habit are known from a hybridization between *P. peltatum* and *P.* ×*hortorum* (COFFIN and HARNEY, 1978) and can be a result of genomic conflict (BOMBLIES and WEIGEL, 2007).

Hybrids from both interspecific combinations developed sterile flowers. Anthers were shrivelled, and no fruit development was observed after pollination with *PTF*. HORN (1994) also reported sterile progeny from the combination *P. peltatum* × *P. acetosum*. Hybrid sterility can be a consequence of either karyotypical differences or genetic incompatibilities (BOMBLIES, 2010). If the hybrid sterility was caused by different karyotypes, a polyploidization could restore fertility and allow further generations on the tetraploid level (SATTLER et al., 2016). Nevertheless, if the sterility was a consequence of genetical incompatibilities, a restoration of fertility would be rather unlikely (RIESEBERG and WILLIS, 2007).

The matte bluish leaf surface of *P. acetosum* did not appear among hybrids from the combinations *AC1* × *PTF* and *PTF* × *AC2*. All plants had leaves with a rather glossy surface comparable to *PTF*. However, the leaf bases of all hybrids were cordate (unlike the peltate leaf base of *PTF*), and leaf zonation was not observed. Leaf zonation is dominant over zoneless leaves in *P.* ×*hortorum* (AMOATEY and TILNEY-BASSETT, 1993), but this does not seem to apply here. Hybrids from the combination *PTF* × *AC2* showed a high susceptibility to thrips, which was most likely inherited from *AC2*.

Information about the inheritance of the waxy leaf surface of *P. acetosum* is limited. In the hybridization between *P. acetosum* and *P.* ×*hortorum* (HONDO et al., 2014), leaves of the F_1 generation showed a rather intermediary level of wax bloom, and hybrids with a leaf surface comparable to *P. acetosum* first appeared in the F_2. In

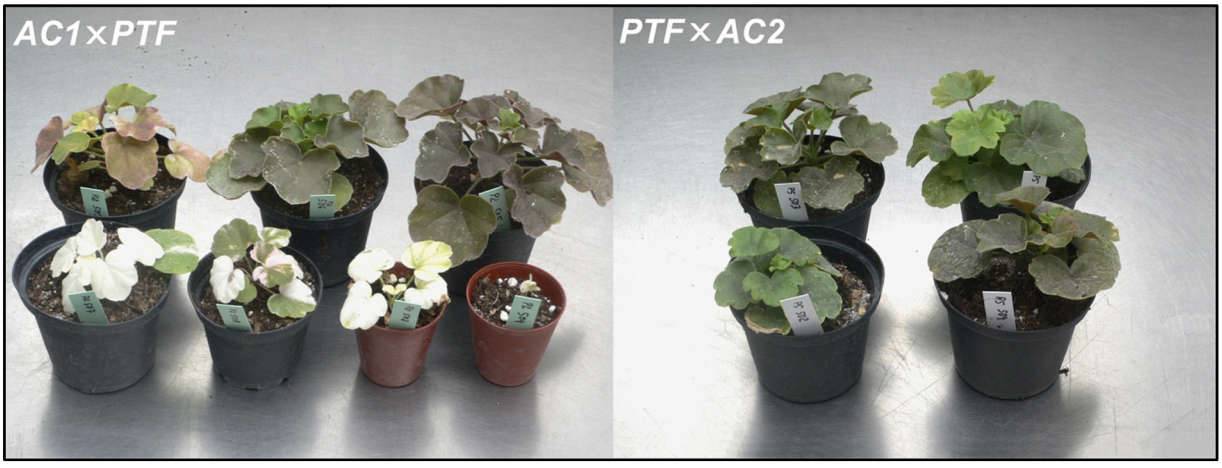

Fig. 3: Progeny from the combinations *AC1* × *PTF* (left) and *PTF* × *AC2* (right), 14 weeks after sowing. Pot diameters: 9 cm, 7 cm, 4 cm (*AC1* × *PTF*), and 9 cm (*PTF* × *AC2*).

order to achieve hybrids with bluish leaves from the hybridization between *P. acetosum* and *P. ×peltatum,* a further generation would be necessary, which seems only possible through previous polyploidization (see above).

Hybrid status was determined based on the above-mentioned morphological traits and was additionally supported by the occurrence of hybrid incompatibilities. Nevertheless, for an unambiguous confirmation of hybridity, a molecular analysis of the hybrid character should be considered, such as AFLP (amplified fragment length polymorphism) or RAPD (random-amplified polymorphic DNA) fingerprinting (Barcaccia et al., 1999; Kuligowska et al., 2016).

Embryo rescue

Two weeks after the establishment of embryo culture, most embryos started forming callus. In the case of *AC2 × PTF*, only six of the 20 cultivated embryos formed callus while the rest died off. Embryos below the torpedo stage did not survive (Scemama and Raquin, 1990). A later start of embryo culture may have increased the number of surviving embryos (Becker-Zens, 1983), as embryos of the combination *AC2 × PTF* were slightly more developed three weeks after pollination (Fig. 1). In contrast, embryos of the other combinations were developed enough to begin embryo culture two weeks after pollination because they were at least in a bent torpedo stage (Fig. 1) (Bentvelsen et al., 1990; Scemama and Raquin, 1990).

From the fourth week after establishment of culture, countless tiny organoids developed on most samples (except for *AC1 × AC1,* which showed organoid formation only on less than half of the samples). Most samples showed substantial callus proliferation. In the seventh week after establishment, an increasing number of adventitious shoots showed signs of vitrification. These symptoms are known from the micropropagation of *P. ×peltatum* on culture media containing BAP (Wojtania and Gabryszewska, 2001; Wojtania, 2010). Some of the samples recovered after transfer to the phytohormone-free culture medium, but only a few adventitious shoots showed substantial elongation. Samples of *AC1 × AC1* showed a particularly stunted growth and consequently senescence of callus and shoots. After transfer of single shoots into jars, shoots of all samples continued growing slowly, and only a few shoots rooted. A strongly inhibitory effect of BAP on shoot elongation of *P. ×peltatum* was documented by Wojtania and Gabryszewska (2001). This effect apparently continued a long time after samples were removed from the BAP containing medium.

As discussed above, embryos and seedlings of the combination *AC1 × PTF* showed chlorophyll deficiencies most likely due to an incompatibility between one of the two plastomes and the hybrid genome. In vitro, samples of this combination had a strong tendency towards a segregation of intact and chlorophyll-deficient tissue. Consequently, many shoot regenerates were completely white and some entirely green (Fig. 4). Samples of the combination *AC2 × PTF* exhibited chlorophyll deficiencies only on intercostal fields of the leaf blade (Fig. 4). Because these symptoms only occurred in vitro, it remains unclear whether these were an effect of genomic conflict or other factors.

As a consequence of low shoot qualities, low rooting rates, and chlorophyll deficiencies, very few plants endured the transplantation of shoots into organic substrate and the following acclimatization to the greenhouse. From the combination *AC1 × PTF*, four genotypes had survived six months later, two of them exhibiting chlorophyll deficiencies and hybrid variegation. From the combination *AC2 × PTF,* plants of only one genotype was successfully relocated to the greenhouse. These had green and glossy leaves and showed a high susceptibility to thrips, comparable to hybrids from the combination *PTF × AC2*. All flowering hybrids deriving from embryo rescue exhibited shrivelled anthers. In this regard, they did not differ from hybrids deriving from seeds. Morphological traits indicating a spontaneous polyploidization have not been observed. The latter is a frequent result of in vitro regeneration (Plaschil et al., 2015).

Conclusion

In the hybridization between *P. acetosum* and *P. ×peltatum,* the occurrence of most hybridization barriers varied strongly between the performed combinations and depended on both the genotype and the direction of cross-breeding. The combination *AC1 × PTF* was hampered by chlorophyll deficiencies and hybrid variegation. Viable hybrids were both achieved from seeds and via embryo rescue, but the latter did not considerably increase the number of hybrids. The combination *AC2 × PTF* was characterized by an incomplete embryo development. In this case, hybrids were only accomplished through the use of embryo rescue. The reciprocal combination *PTF × AC2* appeared to be impeded by prezygotic barriers, and one hybrid was characterized by a permanently stunted plant growth. All flowering hybrids exhibited sterile flowers, and the occurrence of hybrid sterility was independent of both the genotype and the direction of the

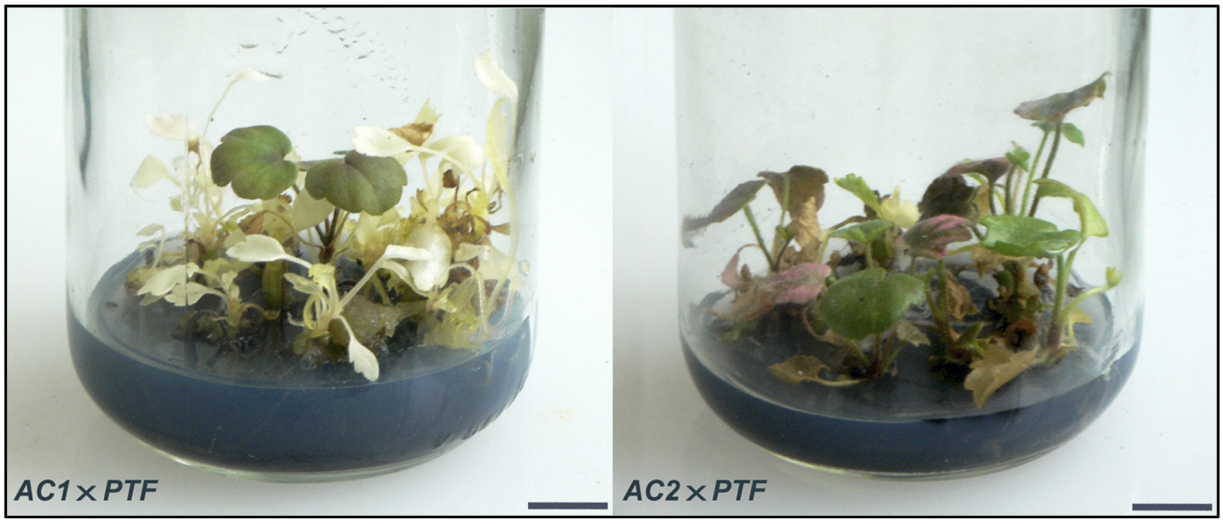

Fig. 4: Shoot regenerates from the interspecific combinations *AC1 × PTF* (left) and *AC2 × PTF* (right), cultured on AC-II-medium (six weeks after transfer). All regenerates within one jar (5.9 cm Ø) originated from the same rescued embryo.

cross. Hybrid sterility represented the major barrier in the hybridization between *P. acetosum* and *P. ×peltatum*. To overcome hybrid sterility a polyploidization would be necessary (Rieseberg and Willis, 2007; Sattler et al., 2016).

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Address of the corresponding author:
R. Kamlah, Lausitzer Platz 1, 10997 Berlin, Germany
E-mail: rainer.kamlah@web.de