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Preparing Interspecific Hybridization in the Genus *Lobelia* – Nuclear DNA Contents, Pollen Handling, and Localization of Crossing Barriers

M. Elansary and T. Winkelmann
(Leibniz Universität Hannover, Institute of Horticultural Production Systems, Section Woody Plant and Propagation Physiology, Hannover, Germany)

Summary

The genus *Lobelia* is composed of 415 species, which are allocated to 18 sections (Lammers 2011). Among the numerous species of the genus, *Lobelia erinus* hybrids have a very high ornamental value. Breeding has resulted in cultivars with colours as white, pink, lavender, purple and blue, while the red colour is missing. Therefore, possibilities for interspecific hybridization with red-flowering species such as *L. gerardii* and *L. cardinalis* were explored to produce a novel red-flowering type of *L. erinus* hybrids. In a first step DNA content and ploidy level in 39 *L. erinus* genotypes and 9 other *Lobelia* species were estimated using flow cytometry and chromosome countings in selected examples. In the *L. erinus* types tetraploid cultivars had DNA contents of 2.24–2.96 pg $2C^{-1}$, and triploid genotypes 1.78–2.11 pg $2C^{-1}$. Secondly, pollen viability was determined by in vitro pollen germination tests and revealed percentages of 39.1–84.7 % for different selected genotypes, but only 0.5–7.8 % for triploid *L. erinus* hybrids. Pollen storage at −20 °C is possible for at least 6 months with only slightly decreased viability. Moreover, the pollen tube growth was examined at 24 and 48 h after pollination by aniline blue staining in 59 crossing combinations. In most of the crosses the pollen tubes reached the proximity of the ovules. Thus, crossing barriers in interspecific crosses between different *Lobelia* species and cultivars can be assumed to be predominantly post-zygotic, although in-growth of pollen tubes through the micropyle has not been observed.

Key words. flow cytometry – *Lobelia erinus* – ploidy level – pollen viability – pollen storage

Introduction

The genus *Lobelia* is considered the second largest of the 84 genera in the family *Campanulaceae*, and the largest of the 29 genera in the subfamily Lobelioideae (Lammers 2007a). Its members differ in growth habit, reproductive biology, flower structure and colour, pollination, fruit type, seed morphology, and chromosome number (Lammers 2007b). Among the numerous species of the genus *Lobelia* there are several species of ornamental interest. *Lobelia erinus* L. is one of those species that belongs to a genetic diversity centre in South Africa. In spite of the long and intensive breeding and although cultivars of *L. erinus* hybrids are very important bedding plants, there is only little known about their breeding history (Olbricht et al. 2011). Only a few reports about interspecific hybridization of *L. erinus* hybrids are available (Olbricht et al. 2011). *L. erinus* hybrids are characterized by different flower colour, morphologies and variation in growth type. Large variation of colours is found in *L. erinus* cultivars such as the blue, purple and white but the red colour is missing. In contrast, many popular *Lobelia* cultivars of other species or hybrids such as the Fan' series (*L.* 'Fan Scarlet', *L.* 'Fan Salmon'), are red in colour and have been produced from interspecific hybridization of *Lobelia splendens*, *Lobelia cardinalis*, or *Lobelia siphilitica*.

In order to pave the way for interspecific hybridizations, knowledge on the ploidy level and DNA contents of the parental genotypes is helpful. Chromosome numbers have been settled for 70 % of the family, but only for 13 % of *Lobelioideae* subfamily. About 75 % of species have chromosome numbers that are multiples of 7, which is considered the basic chromosome number of the genus (Ruas et al. 2001). From this base, several ploidy levels have developed, including tetraploid (2n = 28), hexaploid (2n = 42), octoploid (2n = 56), decaploid (2n = 70) (Lammers 1993). Flow cytometry is an easy and fast way to determine ploidy level and DNA amounts (Ochatt 2008), but has not been applied in *Lobelia* so far.

A second prerequisite for successful interspecific hybridizations is understanding male and female fertility and the temporal and spatial localization of potential barriers. Therefore, pollen viability tests and analyses of pollen tube growth in the style are commonly used methods to gain first insights (Van Tuyl and De Jeu 1997; Winkelmann et al. 2010; Meiners and Winkelmann 2012).

The present study had the following objectives: (i) to estimate DNA contents and determine ploidy levels of different *Lobelia* species and cultivars, (ii) to determine viability of fresh and stored pollen, and (iii) to identify possible barriers in interspecific hybridizations between species of the genus *Lobelia* by examining pollen tube growth *in situ*.

Materials and Methods

Plant material

This study is based on a selection of *L. erinus* hybrids (given by their series name and cultivars coded by numbers), *Lobelia* species and *L. cardinalis* hybrids (Table 1 and Table 2) kindly provided by Westhoff company (Suedlohn, Germany). The conditions used for cultivating these genotypes in the greenhouse were long day (16 h), a heating set point of 16 °C, a ventilation set point of 18 °C and an humidity of about 60 % rF. The fertilizer used was 'FERTY® 3 MEGA' (Planta Düngemittel GmbH- Germany), which contains 18 % N (10 % NO_3, 8 % NH_4), 12 % P_2O_5, 18 % K_2O and 2 % MgO, in a solution of 0.25‰ with every watering.

Flow cytometry and chromosome counting

For isolation of the nuclei, 0.5 cm^2 fresh young leaf tissue of *Lobelia* species was chopped together with leaf tissue of *Pisum sativum* cv. 'Viktoria', 'Kifejtö Borsó' (Genebank Gatersleben accession number: PIS 630; 2C = 9.07 pg), which was used as a reference standard. Sample tissues were chopped with a razor blade in a Petri dish in 0.25 ml extraction buffer (CyStain PI Absolute P; Partec, Muenster, Germany) and incubated for 1 to 2 min. The suspension was filtered through 30 µm CellTrics filter (Partec) in to a sample tube. Then the staining solution (CyStain PI Absolute P; Partec) containing propidium iodide and RNase was added to the nuclei filtrate and incubated for at least 60 min on ice in the dark. The fluorescence of the extract was measured using the CyFlow Ploidy analyser (Partec). Fluorescence was excited at 532 nm using Nd-YAG laser channel. The 2C DNA content of the sample was calculated by the formula: Sample 2C DNA content = (sample peak mean/standard peak mean) × standard 2C DNA content in pg (Doležel and Bartos 2005). At least three replicates of about 500 to 2000 nuclei were measured for each genotype.

For chromosome counting, young root tips about 0.5–1.0 cm in length were harvested from different *Lobelia* types two to three hours after sunrise (*L.* 'Hot 3', *L.* 'Star 3', *L.* 'Fan Scarlet' and *L.* 'Fan Salmon'). First they were treated with cold water (4 °C) for 2 h. Then water was replaced with fixation solution in a ratio 3:1 (ethanol to glacial acetic acid) and samples were stored at 4 °C overnight. Root maceration was carried out in 1 N HCl at 60 °C for 15 min. The maceration solution was replaced by a staining solution composed of 1 % acetic orcein (in 45 % acetic acid) to stain the chromosomes for one to two hours at room temperature. The staining solution was removed and the root tips were washed three to five times until the red colour disappeared. One millimetre of the root tip was transferred to a microscopic slide for analysis. A drop of acetic acid was added on the root tip, and then covered with a cover slip to be squeezed. The chromosome sample was observed under a bright field microscope (Axio Scope.A1, Zeiss, Oberkochen, Germany) at 1000 x magnification.

Pollen viability

Fresh anthers were removed from flowers at anthesis of ten *Lobelia* genotypes (Table 2) during the flowering time in spring 2011 and 2012. Anthers were dried in open Eppendorf tubes at room temperature for 24 h. Pollen viability was determined via *in vitro* germination, in which pollen was incubated on a modified BK (Brewbaker and Kwack 1963) medium containing 100 g l^{-1} sucrose, 200 mg l^{-1} boric acid, 400 mg l^{-1} calcium nitrate, 100 mg l^{-1} potassium nitrate, 200 mg l^{-1} magnesium sulphate, 7.5 g l^{-1} Plant Agar (Duchefa, Haarlem, The Netherlands) at pH 6. The pollen was incubated in Petri dishes at room temperature (20 °C) in darkness for 2 h. Viability was assessed in approximately 500 pollen grains per sample and is given as percentage after observation under a bright field microscope (Axio Scope.A1, Zeiss, Oberkochen, Germany). The pollen was considered viable when the pollen tube length was longer than the diameter of the pollen grain. Three replicates were done for each genotype (Table 2). In parallel, in vitro germination tests were carried out for stored pollen in which anthers of ten flowers from *L.* 'Hot 3', *L.* 'Star 1', *L.* 'Fan Burgundy' and *L. cardinalis*. were collected and dried in Eppendorf tubes, pollen was stored at −20 and 4 °C for 1, 2, 3 and 6 months. After the respective storage time three replicates of 500 pollen grains each were evaluated for viability as described above.

Statistical analysis

Statistical analyses were carried out in R version 3.0.1 (R Core Team 2013). For pollen viability data, a quasi-binomial generalized linear model (McCullagh and Nelder 1989) was fit to the data. It is a generalization of the linear model and assumes a binomial distribution for

Table 1. Nuclear DNA contents of different *Lobelia* genotypes determined by flow cytometry using *Pisum sativum* as reference standard and deduced chromosome numbers

Species/Series/ Cultivar number	n[1]	2n Chromosome number	Nuclear DNA content (pg $2C^{-1}$)		Coefficient of variance [CV] (%)	
			Mean	SD	Mean	SD
L. 'Curacao 1'	2	21	1.83	0.01	3.90	0.98
L. 'Curacao 2'	3	28	2.75	0.07	3.80	0.36
L. 'Curacao 3'	2	21	2.11	0.08	5.75	0.49
L. 'Curacao 4'	3	28	2.57	0.08	4.23	0.25
L. 'Curacao 5'	3	28	2.49	0.06	4.46	0.20
L. 'Curacao 6'	2	28	2.24	0.02	6.35	0.49
L. 'California 1'	3	28	2.55	0.07	5.25	0.21
L. 'California 2'	3	28	2.41	0.10	5.33	0.50
L. 'California 3'	3	28	2.57	0.04	5.53	0.40
L. 'California 4'	3	28	2.50	0.06	4.90	0.60
L. 'California 5'	2	28	2.40	0.01	5.15	0.91
L. 'California 6'	4	28	2.82	0.04	4.42	0.59
L. 'Hot 1'	3	28	2.75	0.22	4.56	1.19
L. 'Hot 2'	3	28(a)	2.43	0.10	5.23	1.16
L. 'Hot 3'	3	28	2.78	0.10	4.53	0.65
L. 'Hot 4'	3	28	2.62	0.12	4.90	0.72
L. 'Hot 5'	3	28	2.44	0.03	5.06	0.05
L. 'Hot 6'	3	28	2.51	0.18	5.23	0.30
L. 'Hot 7'	3	28	2.64	0.17	4.93	0.55
L. 'Hot 8'	3	28	2.48	0.06	5.03	0.83
L. 'Hot 9'	3	28	2.64	0.14	5.06	1.02
L. 'Hot 10'	3	28	2.77	0.02	5.60	0.43
L. 'Hot 11'	3	28	2.60	0.06	5.03	0.90
L. 'Hot 12'	3	28	2.61	0.15	4.80	0.75
L. 'Hot 13'	3	28	2.56	0.08	3.96	0.20
L. 'Hot 14'	3	28	2.62	0.12	4.50	0.81
L. 'Star 1'	3	28	2.78	0.03	4.86	1.00
L. 'Star 2'	3	21	2.07	0.14	6.73	1.70
L. 'Star 3'	3	18	1.80	0.08	5.80	1.32
L. 'Star 4'	3	28	2.40	0.05	4.83	0.55
L. 'Star 5'	3	28	2.44	0.01	5.03	0.49
L. 'Star 6'	3	28	2.55	0.10	4.56	0.80
L. 'Star 7'	3		2.30	0.21	6.10	0.62
L. 'Star 8'	3	28	2.73	0.06	4.83	1.00
L. 'Heaven 1'	3	28	2.66	0.04	4.56	0.32
L. 'Heaven 2'	3	28	2.76	0.04	4.66	1.00
L. 'Fan Burgundy'	3	20	4.52	0.07	4.23	0.80
L. 'Fan Salmon'	2	20	4.50	0.10	3.15	0.91
L. 'Fan Blue'	3	20	4.74	0.26	5.33	0.25
L. 'Fan Scarlet'	3	20	4.62	0.06	7.26	0.20

Table 1. Continued

Species/Series/ Cultivar number	n[1]	2n Chromosome number	Nuclear DNA content (pg $2C^{-1}$)		Coefficient of variance [CV] (%)	
			Mean	SD	Mean	SD
L. siphilitica	3		2.18	0.05	5.36	0.97
L. gerardii	3	14	4.52	0.14	4.80	1.22
L. tupa	3	42(b)	4.57	0.24	4.56	0.28
L. cardinalis	3	14(c)	4.59	0.05	6.26	0.35
L. excelsa	3	42	4.58	0.27	4.72	0.54
L. erinus 'Red Cascad'	2		1.94	0.02	5.45	0.49
L. erinus 'Lilac Cascad'	2		2.96	0.07	5.40	0.28

[1] n is the number of replicates
The grey highlights are the chromosome values determined/verified in this study
a) Olbricht et al. (2011), b) Lammers and Hensold (1992), c) Cooper (1942)

Table 2. Viability (%) of two-days-old pollen, as estimated by in vitro germination tests. Given are means and standard deviations of three replicates. Means marked with the same letter do not differ according to multiple comparisons and compact letter display (Hothorn et al. 2008)

Genotype	Pollen viability (%)	
	Mean	SD
L. 'Hot 1'	53.3 cf	5.1
L. 'Hot 2'	39.1 d	2.6
L. 'Hot 3'	73.1 b	4.7
L. 'Star 1'	66.8 bf	8.8
L. 'Star 2'	0.5 g	0.9
L. 'Star 3'	7.8 defg	9.9
L. 'Fan Burgundy'	75.4 abc	7.9
L. cardinalis	84.8 a	2.4
L. gerardii	66.1 bf	4.7
L. splendens	68.1 be	3.3

the proportions of viable pollen. The quasi-binomial generalized linear model involves a logit-transformation. The model is as follows

$$\log(\pi_i / (1 - \pi_i)) = ß0 + ß1 \times \text{Cultivar}_i + ß2 \times \text{Month}_i + ß3 \times \text{Month}_i^2 + ß4 \times \text{Temperature}_i + ß5 \times \text{Cultivar}_i \times \text{Month}_i + ß6 \times \text{Temperature}_i \times \text{Month}_i + ß7 \times \text{Cultivar}_i \times \text{Temperature}_i$$

The significance of the variables effect was assessed via an F-test. Multiple comparisons of the time points were carried out using the R package 'multcomp' (Hothorn et al. 2008).

The statistical comparison of pollen viability of pollen between different genotypes was carried out by a sandwich covariance estimator which was applied in generalized linear model (Zeileis 2006) and by a heteroscedasticity-consistent HC3 sandwich covariance matrix (Herberich et al. 2010) to cope with heterogeneity of variances and over dispersion.

Crossings and pollen tube growth in situ

For the analysis of pollen tube growth in situ several crossings were performed within and between selected *L. erinus* hybrids which are indicated by their series name and an internal cultivar number and the red *Lobelia* species in summer 2012 (Table 3). Flower buds of maternal parents were emasculated at an intermediate bud stage, at which the anthers were removed from the flowers. The emasculated flowers were covered by perforated bags (Crispac-bag) made of polyethylene. These bags are permeable to air with small perforation of 1 mm in diameter (Baumann Saatzuchtbedarf, Germany) to avoid uncontrolled pollination.

Artificial pollination was performed two days after emasculation when the maternal stigmas began to increase in size to ensure stigma receptivity. Each flower was covered again with plastic bags. Then the carpels were collected at 24 and 48 h after pollination. They were fixed in a fixation solution of a ratio 2:1 (ethanol to propionic acid) for two days. Fixed carpels where washed two times with distilled water and then macerated with 1 M NaOH for 3 minutes at 60 °C. The carpels were rinsed again three times with distilled water and stained in aniline blue staining solution (100 mg aniline blue (Serva) dissolved in 767.6 mg $K_3\ PO_4 \times H_2O$ in 100 ml distilled water) for at least 2–3 h. The stained carpels were put on a slide and bisected with a scalpel, then squeezed with a cover slip and observed under a fluorescence microscope, (Filter Set 02, Excitation G 365, Beam Splitter FT 395, Emission LP 420) (Axio Scope. A1, Zeiss,

Table 3. Pollen tube growth for different interspecific crosses between *Lobelia erinus* hybrids of the 'Hot' and 'Star' series and the red flowering *Lobelia* 48 h after pollination

♀/♂	*L.* 'Fan Salmon'	*L.* 'Fan Scarlet'	*L. gerardii*	*L. excelsa*	*L.* 'Hot 1'	*L.* 'Hot 2'	*L.* 'Hot 3'	*L.* 'Star 1'	*L.* 'Star 3'
L. 'Fan Salmon'	4(5)				4(5)	5(5)	2(5)	4(5)	3(5)
L. 'Fan Scarlet'	5(5)	5(5)			5(5)	5(5)	5(5)	5(5)	4(5)
L. gerardii			5(5)		5(5)	5(5)	3(5)	5(5)	4(5)
L. excelsa				4(5)	0(5)	0(5)	0(5)	0(5)	0(5)
L. 'Hot 1'	4(5)	5(5)	0(5)	0(5)	5(5)				
L. 'Hot 2'	0(5)	3(5)	0(5)	2(5)		4(5)			
L. 'Hot 3'	3(5)	0(5)	4(5)	4(5)			4(5)		
L. 'Star 1'	5(5)	4(5)	3(5)	5(5)		5(5)		4(5)	
L. 'Star 2'	4(5)	0(5)	2(5)	0(5)					
L. 'Star 3'	0(5)	3(5)	3(5)	3(5)					4(5)

The left side number indicates the number of carpels through, which the pollen tube reached the proximity of the ovules. The number between braces indicates the number of replicates. The grey highlights are the controls.

Oberkochen, Germany). Different *Lobelia* species had different style length; therefore, pollen tube growth was not evaluated according to a fixed length, but relative to the style length. Pollen tube growth was classified according to the following criteria: (1) germination of pollen occurs on the stigma, (2) pollen tube growth through first half of the style, (3) pollen tube growth to the ovary, (4) pollen tube growth to the proximity of the ovules.

Results

Flow cytometry and chromosome counting

Among all analysed species, the nuclear DNA content ranged between 1.83 and 4.74 pg $2C^{-1}$, with standard deviations of 0.01 to 0.26 (Table 1). The CV values for all the sample peaks ranged from 3.15 to 7.46. It was shown from the data in Table 1 that the red flowering types (*L.* 'Fan Burgundy', *L.* 'Fan Scarlet', *L.* 'Fan Salmon', *L. gerardii*, *L. cardinals*, *L. tupa* and *L. excelsa*) had a DNA content which ranged between 4.50 and 4.74 pg $2C^{-1}$. In the 'Hot' Series of *L. erinus* hybrids the DNA content of *L.* 'Hot 1', *L.* 'Hot 2' and *L.* 'Hot 3' was 2.75, 2.43, and 2.78 pg $2C^{-1}$, respectively, which indicates relative similarities in DNA content between cultivars of the 'Hot' series. On the other hand in the 'Star' series the DNA content of *L.* 'Star 1', *L.* 'Star 2', *L.* 'Star 3' was 2.78, 2.07, and 1.80 pg $2C^{-1}$, respectively, pointing to more pronounced differences in DNA content in this series.

Chromosomes were counted for seven *Lobelia* species and cultivars, which represent four different *Lobelia* groups. The analysed cultivars are *L.* 'Fan Scarlet' and *L.* 'Fan Salmon', *L.* 'Star 3' and *L.* 'Star 1', *L.* 'Hot 3', *L.* × *gerardii* 'Vedrariensis' and finally *L. excelsa*. In case of *L.* 'Star 3' the chromosome number was 2n = 18 (Fig. 1A).

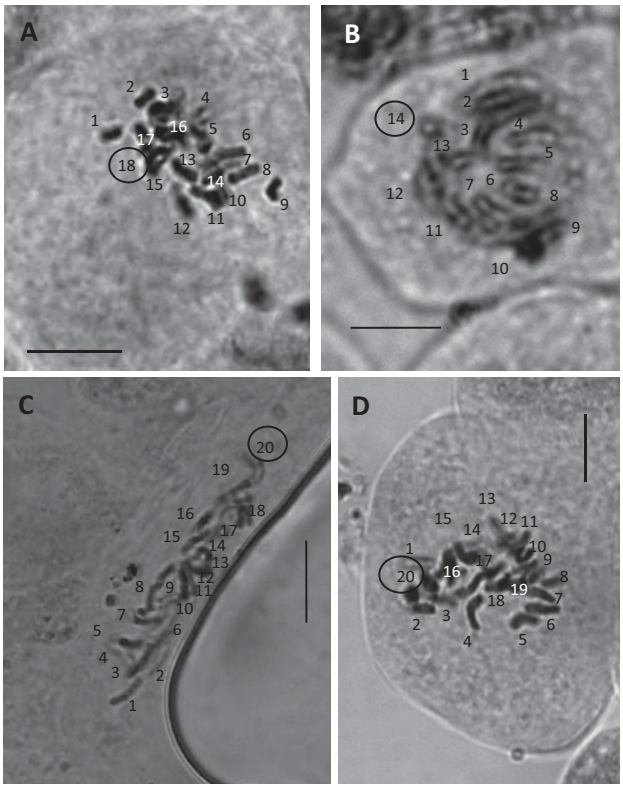


Fig. 1. Chromosome counts from root tips of *Lobelia* species and cultivars stained with aceto orcein: A: *L.* 'Star 3', B: *L.* × *gerardii* 'Vedrariensis', C: *L.* 'Fan Scarlet', D: *L.* 'Fan Salmon'. Numbers in the sub-figures represent chromosome count and the highlights represent the highest figure. Bars represent 10 µm.

Based on the basic chromosome number of the genus *Lobelia* x = 7 (Ruas et al. 2001), for the cultivar 'Star 3' cultivar a chromosome number of 2n = 21 cannot be

excluded, so more metaphase cells should be observed in future. *L.* × *gerardii* 'Vedrariensis' had a chromosome number of 2n = 14 (Fig. 1B). However, in case of *L.* 'Fan Scarlet' and *L.* 'Fan Salmon' in two root tip cells 2n = 20 chromosomes were counted and in one cell 2n = 21 (Fig. 1C, D). For *L. excelsa* a chromosome number of 2n = 40 was recorded, but based on literature *L. excelsa* is a hexaploid (Lammers and Hensold 1992), suggesting a chromosome number of 2n = 42. *L.* 'Star 1' had a chromosome number of 2n = 28, but also based on the basic chromosome number of the genus *Lobelia* x = 7, this cultivar can be assumed to have a chromosome number of 2n = 28. Also *L.* 'Hot 3' had a chromosome number of 2n = 28.

Pollen viability and storage

The first barriers that may hamper interspecific hybridizations are low fertility in male or female gametes. For *Lobelia* pollen, pollen germination *in vitro* began after two hours. By applying *in vitro* germination assay, the viable pollen was differentiated from the dead pollen by the presence of the pollen tube (Fig. 2). Between the ten examined genotypes, significant differences in pollen viability were observed (Table 2), in which eight of the examined *Lobelia* types had relatively high pollen viability which ranged between 40 and 80 %. However, two *L. erinus* hybrids ('Star 2' and 'Star 3') had very low pollen viabilities of 0.5 and 7.8 %, respectively (Table 2).

In the pollen storage experiment, (Fig. 3), based on the quasi-binomial generalized linear model, it was shown that the pollen viability differed significantly between cultivars ($p = 3.46 \times 10^{-9}$), over time ($p = 3.26 \times 10^{-12}$) as well as between the different temperatures (4 °C and −20 °C: $p = 5.22 \times 10^{-9}$). However, no significant difference in change of pollen viability over time for the different cultivars was detected (p = 0.0952).

For all four species the viability of pollen after 2 days drying at 20 °C was between 66 % and 84 %) (Fig. 3). However, the pollen viability decreased after storing the pollen for 6 months at 4 °C (43–45 %) (Fig. 3). On the other hand a temperature of −20 °C was more suitable for long-term storage because after six months of pollen storage viable pollen was detected in a percentage of 56 to 81 % (Fig. 3). Significantly lower viability compared to the two-days old pollen was observed after one to three months of storage at 4 °C, but only after three to six months at the lower storage temperature.

Observation of pollen tube growth in situ

In order to localize pre- and post-zygotic crossing barriers between different *Lobelia* types, the pollen tube growth was observed for the cross combinations listed in Table 3. First, pollen tube growth was detected for selected crosses 24 and 48 h after pollination to spot the time of fertilization. After 24 h most of the pollen tubes had grown through the whole style. After 48 h some of the pollen tubes reached the ovaries, but in most cases they reached the proximity of the ovules. Examples of images that show the pollen germination on the stigma, and pollen tube growth up to the ovules are shown in Fig. 4 for interspecific crosses of *L.* 'Fan Scarlet' with *L.* 'Hot 1' 48 h after pollination.

Pollen tube growth of 59 crosses was summarized in Table 3. Five carpels were evaluated for each cross combination. Pollen tube growth for each of the interspecific crosses was compared to intraspecific crosses within the *L. erinus* hybrids. Pollen tube growth was observed in the proximity of the ovules in most of the crosses, suggesting

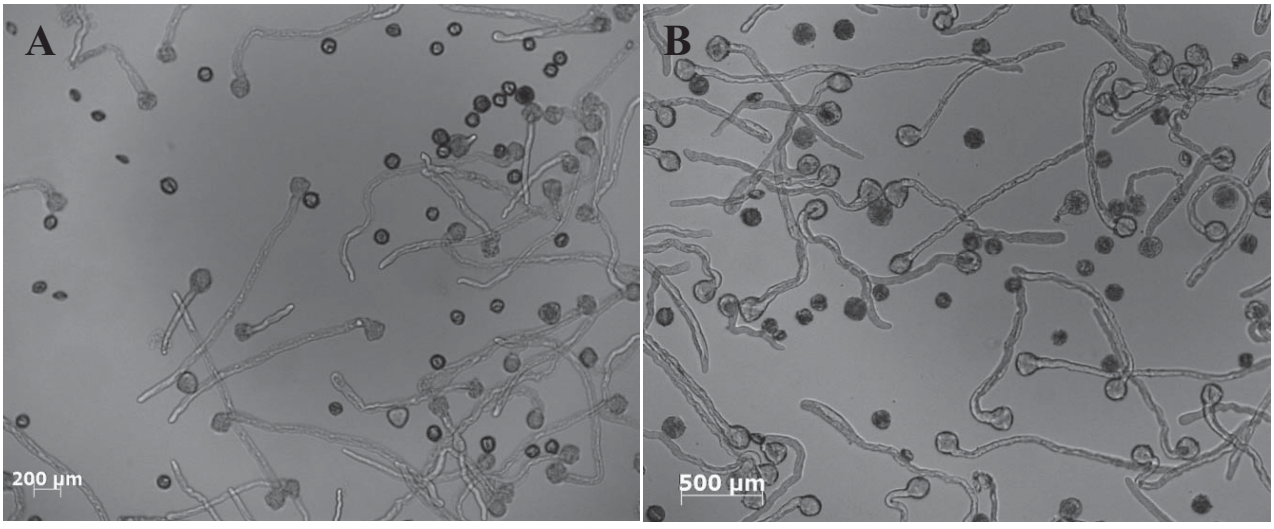


Fig. 2. Pollen tube germination after 2 h incubation on a modified BK medium (Brewbacker and Kwack 1963). A: *L.* 'Fan Burgundy', B: *L.* 'Star 1' after 6 months of storage at −20 °C.

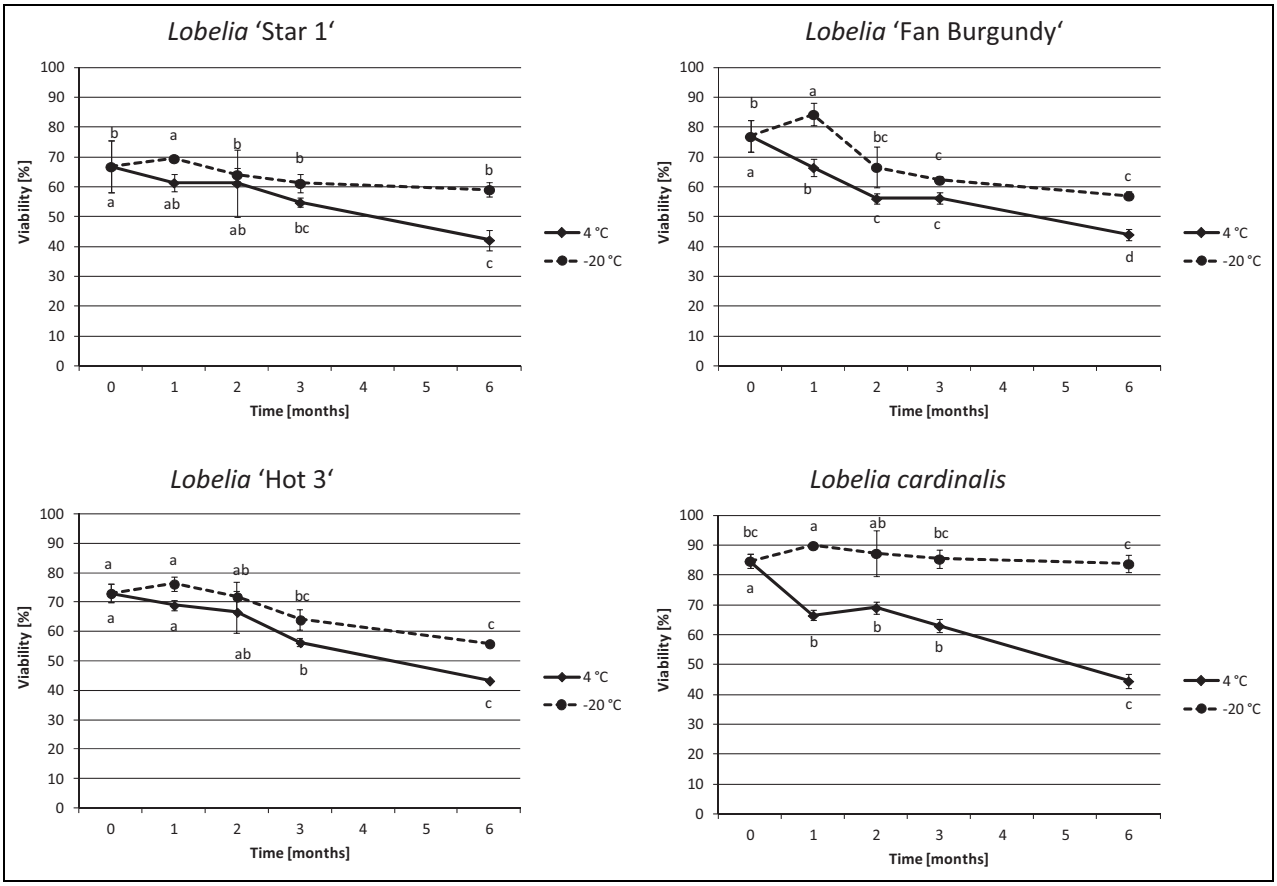


Fig. 3. Viability of pollen stored at 4 °C and –20 °C for six months of four different *Lobelia* types determined by *in vitro* germination method. Data are represented as mean ± standard deviation of three measurements. The values of time 0 refer to the mean of viability percentage of two-days-old pollen for each *Lobelia* type. Letters refer to multiple comparisons of pollen viability at different time points.

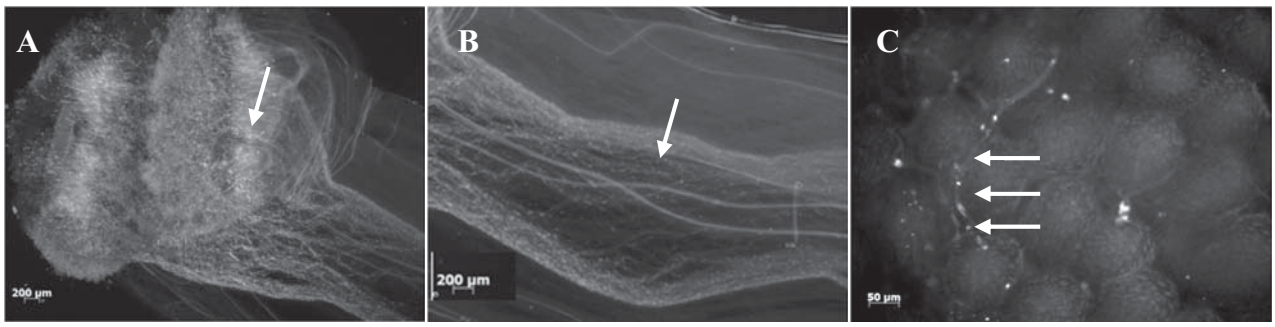


Fig. 4. Pollen germination on the stigma and pollen tube growth through the style to the ovules after interspecific crosses of *Lobelia* 'Fan Scarlet' with *L.* 'Hot 1'. A: The stigma and style, B: The style and C: Ovules 48 h after pollination. The arrows indicate the pollen tube growth.

that fertilization is possible. In Table 3 nine of the crossings are selfings in which the pollen tube succeeded to reach the proximity of the ovules in at least four of five carpels. In the intraspecific crosses, pollen tube growth was detected near the proximity of the ovules in all five carpels (Table 3).

On the other hand, in some crosses the pollen tubes were arrested in the style. This happened in most cross combinations with *L. excelsa* as the mother plant, and also in the crosses between *L.* 'Hot 2' × *L.* 'Fan Salmon', *L.* 'Star 2' × *L.* 'Fan Salmon', *L.* 'Hot 1' × *L. excelsa* and *L.* 'Star 2' × *L. excelsa*. However, in the reciprocal cross when using

the *L. excelsa* as the father plant, the pollen tube was detected in the proximity of the ovules in at least three carpels except when using *L.* 'Hot 1' and *L.* 'Star 2' as maternal parent. In summary, 59 different cross combinations (Table 3) were evaluated for pollen tube growth 48 h after pollination. For 42 crosses, pollen tubes reached the proximity of the ovules in at least three analyzed carpels, indicating that fertilization maybe possible and pointing to predominant post-fertilization barriers.

Discussion

Flow cytometry and chromosome counting

In this study, 48 *Lobelia* species and cultivars have been examined to estimate their DNA content by flow cytometry (Table 1) and six cultivars and one species were examined for their chromosome number. *L. excelsa* had a chromosome number of 2n = 6x = 42, and in agreement with literature this species was interpreted as hexaploid (Lammers and Hensold 1992), which indicates that it could have originated by polyploidy, based on the basic chromosome number of genus *Lobelia* which is (x = 7) and it has a DNA content of 4.58 pg $2C^{-1}$ (Table 1). In the 'Hot' series, the DNA content of *Lobelia* cultivars was relatively stable, ranging between 2.43 and 2.78 pg $2C^{-1}$ (Table 1). These contents were compared to DNA content of species with known chromosome numbers. The DNA content of the tetraploid *L.* 'Hot 3' was 2.43 pg $2C^{-1}$, which was consistent with data previously described in literature for a cultivar in the same series (*L.* 'Hot White'), which had a chromosome number of 2n = 4x = 28 (Olbricht et al. 2011) and a DNA content of 2.78 pg $2C^{-1}$ (Table 1). Therefore, the chromosome number of *L.* 'Hot 1' could be proposed to be 2n = 4x = 28 and the same can be applied for the rest of the cultivars in the 'Hot' series (Table 1).

While in case of the 'Star' series, there was a pronounced variation in the DNA content and the chromosome number of the cultivars. For example *L.* 'Star 3' had a DNA content of 1.80 pg $2C^{-1}$ and a chromosome number of 2n = 18 (Table 1). Problems in male gamete formation in this *Lobelia* cultivar, were also proven by the low pollen viability (Table 2), while *L.* 'Star 1' had a DNA content of 2.78 pg $2C^{-1}$ and a chromosome number of 2n = 4x = 28 (Table 1). Based on the DNA contents in Table 1, it can be predicted that the chromosome numbers for *L.* 'Star 4', *L.* 'Star 5', *L.* 'Star 6' and *L.* 'Star 8', which had nearly the same DNA content as *L.* 'Star 1', is 2n = 4x = 28, while the chromosome number for *L.* 'Star 2' could be 2n = 3x = 21. However, it is difficult to predict the chromosome number of *L.* 'Star 7' which had a DNA content of 2.3 pg $2C^{-1}$ (Table 1). The same can be applied for the other two series of *Lobelia* cultivars ('Curacao' and 'California') (Table 1).

Variation in DNA content of cultivars of the same series may be explained by the selection of these genotypes just based on phenotypic parameters, but not on genetic relationship.

The coefficient of variation (CV) of the flow cytometric analyses indicates the sharpness of the peak and assesses the accuracy of individual measurements but it is not related to the reproducibility of DNA contents. It is calculated as standard deviation/Peak mean × 100 % (Doležel and Bartos 2005). The CV values among all examined species and cultivars ranged from 3.15 % to 7.46 % (Table 1). The appropriate ranges for CV values are stated by Doležel and Göhde (1995, cited in Galbraith et al. 1998), in which the histograms with peak CVs below 3 % are considered fully acceptable. The authors also state that this may not be achievable in 'difficult' species, where CVs below 5 % are also considered acceptable.

In case of the 'Fan' series, the DNA content ranged between 4.50 and 4.74 pg $2C^{-1}$ (Table 1) which are considered to be similar. The chromosome numbers for *L.* 'Fan Salmon' and *L.* 'Fan Scarlet' was 2n = 20 (Fig. 1C, D). There is no data in literature that supports these chromosome numbers. So, there could be several explanations for this. First, the chromosome number of *Lobelia* 'Fan' series could be based on another basic chromosome number (e.g., x = 10), so by this they could be considered as diploids. Second, an incorrect counting of chromosome number could have occurred due to overlaying chromosomes. Third, an aneuploidy could have occurred leading to the loss of one chromosome. Therefore, they could be triploids of 2n = 21. However, they are fertile. This could be explained if viable gametes would contain the haploid or diploid number of chromosomes if there was a constant pattern of univalent distribution. Further studies should be done to determine the exact chromosome number for the 'Fan' series and to determine the ploidy level of their parents.

In this study, flow cytometry offered a good method to detect the ploidy level of different *Lobelia* species and cultivars and it helped in excluding triploids. However, it is not possible to substitute the classical method of chromosome counting by flow cytometric analysis, because using flow cytometry technique has some limitations. In some cases, it is difficult to isolate sufficient intact nuclei for analysis, which mainly depends on the structure of the sample. Fluorescent debris particles may aggregate to the surface of the isolated nuclei, which consequently results in DNA histograms of low resolution. Moreover, the interference of cytosolic compounds or any secondary metabolites with fluorescent dyes of DNA, may lead to a decrease in the fluorescence intensity (Doležel et al. 2007).

Pollen viability and storage

The pollen viability of two-days-old pollen was relatively high (60–84 %) (Table 2) except for two *Lobelia* species: *L.* 'Star 2' and *L.* 'Star 1' which had extremely low germination ability. This low viability was explained by their ploidy level as they were found to be triploids or aneuploids.

When drying and storing the pollen for six months at 4 °C the viability decreased slightly in comparison to two-days-old pollen (Fig. 3). A reason for this could be the deficiency of respiratory substrates due to the continued metabolic activity of the pollen, even though this activity is going on at a reduced rate (Willson et al. 1979). Linskens and Pfahler (1973) reported remarkable changes in the carbohydrate and organic acid content of the pollen of various maize genotypes during long-term storage.

On the contrary when drying and storing the pollen at −20 °C for six months, pollen viability remained relatively high (61–90 %) (Fig. 3). Perveen (2007) examined the pollen viability of *Pisum sativum* L. after storing the pollen at 4, −20, −30 and −60 °C. The percent of viability was 42, 45, 48 and 60 % respectively after 48 weeks. The pollen showed high viability between −30 and −60 °C. Barnabás et al. (1992) found that storing pollen of different species at sub-zero temperatures resulted in a gradual decrease in pollen viability due to reduced metabolic activity in pollen. Thus subzero conditions seem to show success for the long term preservation of pollen. Moreover, pollen storage is a useful method for pollinating genotypes that flower at different times.

In conclusion, storing pollen grains for six months at −20 °C was the most suitable condition for maintaining pollen viability in *Lobelia*.

Pollen tube growth in situ

Pollen tube growth *in situ* is a special behaviour for plant cells, as it relies on the biochemical and physical interaction of the male gametophyte of the pollen donor plant and the stigma tissue of the receptive flower.

In interspecific crosses between *L. erinus* hybrids and the red flowering *Lobelia*, in most cases pollen tubes reached the ovules after 48 h from pollination. However, in some cases pollen tubes were observed in the style but did not complete the way up to the ovary and the ovules. This case was mainly in the crosses were *L. excelsa* was used as the mother plant (Table 3). An explanation for this could be that *L. excelsa* has a rather long style while the other *L. erinus* hybrids are of short style, so the failure of pollination may have occurred due to intrinsic inability of pollen tubes to grow beyond the length of their own pistils, so they were arrested in the style (Shivanna 1997). However, in most of the reciprocal crosses when using *L. excelsa* as the father plant the pollen tube successfully reached the proximity of the ovules in at least three carpels (Table 3), There are many examples e.g. *Nicotiana* and *Lilium* (Shivanna 1997), *Datura* (Blakeslee et al. 1959), *Vigna* (Gopinathan et al. 1986) in which the length of the style in the two parental species was significantly different. As a result, the crosses were only successful in one direction. This type of pre-fertilization barrier is known as unilateral incompatibility (UI) (Shivanna 1997). Unilateral incompatibility occurs when pollen tubes reach and fertilize the ovules in one direction cross, but is inhibited in the stigma, style or ovary in the reciprocal crosses (Nacionus and Pickerscill 2004). Besides differences in style length in most cases it was due to the lack of mutual adaptation between the pollen and the pistil. Therefore, inhibition of pollen in this case resulted from the lack of genetic information in one parent for relevant character of the other parent and this is called 'incongruity' (Hogenboom 1973).

The pollen tube was also arrested in the style of other crosses such as in the cross between *L.* 'Hot 3' × *L.* 'Fan Salmon' (Table 3). This could be also due to the inability of the pollen tubes to utilize stylar nutrients, which may be due to lack of suitable nutrients in the transmitting tissue or lack of suitable enzymes in the pollen tubes (Shivanna 1997). However, in the reciprocal cross the pollen tube successfully reached the proximity of the ovules in at least three carpels (Table 3). In some cases the pollen tube reached the ovary, but did not reach the proximity of the ovules such as in the cross between *L.* 'Star 3' × *L.* 'Fan Scarlet', *L.* 'Hot 1' × *L.* × *gerardii* 'Vedrariensis' (Table 3). The inhibition of the pollen tube in the ovary may be due to the lack of time for pollen tube to reach the ovules. However there are many examples of this phenomenon such as in the cross between *Maize* × *Sorghum* (Heslop-Harrison et al. 1985). The ability of pollen tube to enter to the proximity of the ovules seems to be dependent on the presence of a chemotropic substance in the micropyle, so failure of the pollen tube to enter the micropyle may be due to the absence of that substance (Heslop-Harrison et al. 1985).

In conclusion, the results and observations of the examined crosses (Table 3) have shown that in most of the analysed carpels, pollen tubes reached the proximity of the ovules, signifying that fertilization can occur. However, no seed set was obtained from any of the interspecific crosses indicating that the crossing barriers between *Lobelia* species are mainly post-fertilization barriers, although successful fertilization could not be proven in this study. In future studies embryo rescue experiments could be carried out in order to overcome this type of barriers.

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Addresses of authors: M. Elansary and T. Winkelmann (corresponding author), Leibniz Universität Hannover, Institute of Horticultural Production Systems, Section Woody Plant and Propagation Physiology, Herrenhaeuser Str. 2, 30419 Hannover, Germany, e-mail (corresponding author): Traud.winkelmann@zier.uni-hannover.de.