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Application of Mixed Incongruous Pollen for Interspecific Crosses of Lilies

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ABSTRACT

Creating interspecific hybrids between distantly related species is the first critical step in breeding of lilies. Different methods are used to overcome pre-fertilization and post-fertilization barriers in incongruous interspecific crosses of lilies. Compatibility between female pistil and pollen play an important role on fertility of crosses. Results are presented showing that pollen incongruous to female pistil can perform fertilization if they are used in mixtures with other incompatible pollen. To overcome pre-fertilization barriers cut-style pollination and pollination of stigma by mixed incompatible pollen were performed. In some cases recalcitrant crosses were more efficient when stigma was pollinated by mixed pollen of incongruous species than when cut-style was pollination by separated pollen of one incongruous male. However, cut-style pollination was efficient in cases when long style possessing females were pollinated by short style possessing male species. Though it is known that crosses of Asiatic hybrid (Asiatic hybrids (A) with *Lilium longiflorum* or Oriental hybrids (O) possible perform only in one direction where Asiatic hybrid participate as male, the pollen of *L. longiflorum* and O stimulated fertilization of Asiatic hybrid when they were added in mixtures with pollen of *L. candidum*, *L. henryi*, *L. monadelphum* or Trumpet and Aurelian hybrids (T). However, any stimulation of fertilization was observed when pollen of *L. longiflorum* and O were added in mixtures with pollen of *L. concolor* or *L. pumilum* to pollinate native pistil of Asiatic hybrid. Pollination of Asiatic hybrid by mixed pollen of incongruous species resulted progeny among which hybrids Asiatic hybrid × *L. regale* and Asiatic hybrid × *L. candidum* were screened by inheritance of molecular markers characteristic to male species.

Keywords: *Lilium* ssp., interspecific hybridization, pre-fertilization barriers, RAPD, SOD
Abbreviations: AC, Asiatic hybrid × *Lilium candidum* hybrids; **AR**, Asiatic hybrid × *L. regale* hybrids; **Asiatic hybrid**, Asiatic hybrids; **C**, *L. candidum*; **Con**, *L. concolor*; **H**, *L. henryi*; **L**, *L. longiflorum*; **LH**, Longiflorum hybrids; **M**, *L. monadelphum*; **OH**, Oriental hybrids; **P**, *L. pumilum*; **PCR**, polymerase chain reaction; **R**, *L. regale*; **RAPD**, randomly amplified polymorphic DNA; **SOD**, superoxide dismutase; **TH**, Chinese Trumpet and Aurelian hybrids

INTRODUCTION

The economically important groups of lilies Asiatic hybrids (Asiatic hybrid), Oriental hybrids (O) and Longiflorum hybrids (L) are derived from interspecific hybrids of phylogenetic related species inside sections Sinomartagon, Archelirion and Leucolirion respectively. Since reproduction isolation barriers have been one of the main criteria determining species and limit exploring interspecific hybrids in breeding programs, different methods to overcome incongruity at pre- and post-zygotic stage have been developed (Asano and Myodo 1977; Van Tuyl *et al*. 1991; Chi 2000, 2002). It resulted in new groups of lilies combining integrated traits of Asiatic hybrids, Oriental hybrids and *L. longiflorum* (OA, LA, OL) (Van Tuyl *et al*. 2002; Lim *et al.* 2008; Van Tuyl and Arens 2011). However, many species of genus *Lilium* possessing valuable traits are yet to be involved in breeding programs (Wang *et al.* 2009).

Inhibition of pollen tube growth along style is critical factor preventing fertilization and participates in creating pre-zygotic isolation barriers between species. By the other point of view the self incongruity between female sporophyte and male gametophyte acts as mechanism preventing self-pollination in cross-pollinated species. The methods to avoid inhibiting effect of style on growth of pollen tube and to overcome this pre-zygotic isolation barrier may be distributed conditionally into two groups: 1) the methods allowing the step of pollen tube growth through an incompatible style and 2) the methods based on physiological and biochemical changes which effect interaction between style and pollen tube. The methods of the first group are: cut-style pollination (Asano and Myodo 1977; Van Tuyl *et al.* 1991); pollination of a grafted style which is compatible to pollen (Van Tuyl *et al.* 1991) and pollination of isolated ovules (Chi 2000). The methods of the second group are: treatment of style by organic solvents (Willing and Pryor 1976) or immunosuppressors (Mujeeb-Kazi 1981); heat treatment of style (Ascher and Peloquin 1968) or performing crosses at high temperatures (Van Tuyl *et al*. 1982; McRae 1998); pollination of aged flower (Ascher and Peloquin 1966) and applying for pollination pollen of incompatible species mixed with viable compatible (Brown and Adwilaga 1991) or mixed with inactivated compatible pollen (Van Tuyl *et al.* 1982: Van Creij *et al.* 2000).

All these methods have one's own merits and faults and success of employment in each case depends on biological peculiarities of plant species. Cut style pollination technique allows circumvent stigmatic and stylar barriers arresting pollen tube growth and is widely employed to perform incongruous crosses of lilies (Asano and Myodo 1977: Van Creij *et al*. 1993). However, cut style pollination results low seed set since pollen tube prematurely penetrates ovary (Janson *et al*. 1993). Placental pollination or pollination of isolated ovules is related with the same problem (Van Creij *et al.* 1993; Chi 2000). The grafted style pollination method allows dissolve problem of premature arrival of pollen tube

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to ovule, however creatures others. The compatible grafted style must be joined to ovary very precisely and despite the fact that grafted style method gives a better seed set it was not successful in a high (Van Tuyl *et al*. 1991). The use of mixed pollen when pollen of incongruous species is mixed with viable compatible pollen or the "mentor" pollination method, when inactivated compatible pollen is mixed with incongruous pollen are used to perform incongruous crosses. However, pollination by mixed pollen tends give very low percent of hybrid progeny (Song *et al*. 2002). Though "mentor" method allowed perform incongruous crosses between some plant species (Nassar *et al*. 1996) for lilies it was effective only in overcoming self incompatibility but not for interspecific crosses (Van Tuyl *et al*. 1982; Van Creij *et al*. 2000). If do not go in details of biochemical mechanisms of incongruity some facts allow predict that mixed pollen can not only support but perhaps rivals each other during pollen tube growth throw style. It was shown that adding pollen of alien species to self incompatible pollen allowed receive selfed progeny in self incompatible species of *Helianthus* and resulted in a very low percent of interspecific hybrids (Desrocers and Riesenberg 1998). This finding has implications for sunflower breeding experiments and provides a simple method for generating selfed plants. Since many species and cultivars of lilies are self incompatible it should be interesting to adopt this method for selfing of lilies. Interspecific crosses of two species can be facilitated by applying to the pollination of one species mixed pollen of several varieties of the other species. Such pollination method was used long ago to perform interspecific crosses in breeding of fruit trees (Yenikyev 1965). J. L. Freeman used mixed pollen of different varieties of Asiatic hybrid to realise interspecific crosses with *L. lankongense* (McRae 1998). If presume that penetration of pollen tubes throw style acts by rival principle it is possible to expect that among mixed incongruous pollen of several species some should participate in fertilization. We apply mixed incongruous pollen of several distant species (*L. candidum*, *L. monadelphum*, *L. regale*, *L. longiflorum*, *L. henryi*) and cultivars from distinct groups (T, O) to pollinate Asiatic hybrids. Pollination by mixed incongruous pollen resulted in progeny with random paternity among which the hybrids Asiatic hybrid x *L. regale* and Asiatic hybrid x *L. candidum* were screened. However, incongruous pollen of other species did not stimulate fertilization of Asiatic hybrid by pollen of *L. pumilum* and *L. concolor*.

MATERIALS AND METHODS

Plant material

The next cultivars, hybrids and species of *Lilium* were used in this research: 1) Asiatic hybrids (Asiatic hybrid) - 'Colleen', 'Disco', 'Lollypop', Red Carpet', 'Rosita', 'Montreux', 'Toscana', 'Vostoc 2', 0-42-1 [('Rozovaja Dymka' × 'Symfonieta') × 'Marlene'], 0-26-4 [('Zhizel' × 'Sancerre') x 'Reinesse'], 0-22-1 and 0-22-13 ('Bajaderka' × 'Symfonieta') F_1, 0-49-1 [('Bajaderka' × 'Symfonieta') × 'Pink Chique'], JP-1 ('Rozovaja Dymka' × 'Symfonieta'); 2) Chinese Trumpet and Aurelian hybrids (T) - 'Golden Splendor', 'Pink Perfection'; 3) Oriental hybrids (O) - 'Acapulco', 'Sorbonne', 'Muscadet'; 4) species - *Lilium longiflorum* cv. 'Snow Queen', 'White Heaven'; *L. candidum* L., *L. monadelphum* Bieb., *L. regale* Wils., *L. henryi* Baker, *L. pumilum* DC, *L. concolor* Salisb. var. *stictum* Stearn.

Pollination trials, embryo rescue and pollen germination test

Plants were grown in greenhouse in standard condition for lily growth. Flowers were emasculated 1 day before anthesis. At least ten flowers were pollinated in each crossing combination (**Table 1**). For pollination, the pollen was applied on the stigma or on the surface of a cut style suspended in a solution of 10% sucrose and 0.02% boric acid in water. For the cut-style method, the style was cut 1-2 mm above the ovary. For mixed pollination incongruous pollen were mixed in equals parts before crossing.

The embryos were isolated 30 days after pollination from ovules of swelled ovary and cultured *in vitro* on solid MS medium (Murashige and Skoog 1962) supplemented with 3% sucrose at 25°C in dark. After germination plantlets were grown in light and later transplanted in pot with peat under greenhouse conditions.

Mature fresh pollen was suspended in drop of solution with 10% sucrose and 0.02% boric acid on the objective slide and incubated at 25°C to perform test of germination. Slides with germinating pollen were contained in Petri dish with wet cotton to avoid evaporation of solution After 10-12 h, the germinated pollen was counted.

Cytogenetic analysis

The root tips of progenies were treated with 0.01% colchicine (SIGMA-ALDRICH, Steinheim, Germany) solution at 4°C during 17 h and subsequently fixed with an acetic acid and ethanol (1:3) mixture. The fixed root tips were stored in 70% ethanol in a freezer until used. Dried root tips were immersed in 4% $(Fe(NH_4)(SO_4)\cdot 12H_2O$ for 2 min and stained 7 min in hot solution of acetocarmine. After cooling root tips were softened by chloral hydrate. The metaphase chromosomes were analysed through a microscope (Nicon Eclipse 80*i*, Japan).

Screening of interspecific hybrids

1. Superoxide dismutase assay

Superoxide dismutase (SOD, EC 1.15.1.1) electrophoretic fractions were analyzed from leaves of plants grown 4 weeks *in vitro*. For this, 0.5 g of leaves was homogenized in 1 ml of potassium phosphate buffer (pH 7.8). After extraction, samples were centrifuged at 4°C for 15 min. After centrifugation 25 ml of supernatant was used for isoelectric focusing in 10% polyacrylamide gels (PAAG). Activity of SOD isozymes was detected by tetranitrotetrazol blue (NBT) reduction (Beuchamp 1971).

2. Random amplified polymorphic DNA (RAPD) analysis

Crude genomic DNA was extracted using a modified CATB method (Murray and Thompson 1980) from 200 mg of leaves harvested from plantlets grown four weeks *in vitro*. Polymerase chain reaction (PCR) was performed, using a thermal cycler (Biometra T personal), in 25 ml reaction mixtures containing 30 ng of template DNA, 1 × reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 units of *Taq* DNA polymerase (Fermentas, Lithuania) and 0.5 mM random primer. Random primers Y29 (5′– TTC GGG CCG T – 3′), Y35 (5′– TGG TAT CAG AGC C – 3′) and Y37 (5′– GTC AAG CGC G – 3′ (BIOMERS, Göttingen, Germany) were used. After an initial heating at 94°C for 3 min. the reaction mixtures were subjected to amplification for 20 cycles each of which consisted of 30 s at 94°C, 1 min at 34°C, 1 min at 72°C, followed by 10 min at 72°C. Amplification products were electrophoresed on 1.5% agarose gels with TBE buffer. Gels were stained with 2.5 mg/ml ethidium bromide and visualized by 300 nm UV light.

RESULTS AND DISCUSSION

Incongruity between Asiatic hybrids and other species of *Lilium*

Several times we tried to receive hybrids between Asiatic hybrid and such species as *L. candidum*, *L. regale* and *L. monadelphum* by routine pollination of stigma. Asiatic hybrid cultivars 'Colleen', 'Disco', 'Lollypop', 'Red Carpet', 'Rosita', 'Montreux', 'Toscana', 'Vostoc 2', and seedlings 0-42-1, 0-26-4, 0-22-1 and 0-22-13, 0-49-1, JP-1 were used to perform crosses with *L. candidum*, *L. regale* and *L. monadelphum* in both reciprocal directions: in some cases Asiatic hybrid participated as female and in other cases as male partner (**Table 1**). Crossing combinations *L. monadelphum* × Asiatic hybrid were fertile and set numerous well developed seeds which germinated without embryo rescue techniques. However, progeny of such combinations looks

Table 1 Pollination methods and fertility of interspecific crosses.

Crossing combinations*	Amount of viable embryos per 1 pollinated flower		Amount of obtained offspring
	By stigma pollination	By cut style pollination	
A × C	0	0	0
A × M	0.01 ± 0.01	0	3
A × R	0.01 ± 0.01	0	2
A × TH	0	0	0
A × L	0	0	0
A × O	0	0	0
A × H	0	0	0
A × mix (C, M, R)	7.9 ± 3.6	-	63
A × mix (C, R)	0.5 ± 0.3	-	12
A × mix (C, L)	2.8 ± 1.5	-	11
C × A	0	0	0
C × L	0	0	0
C × mix (A, L)	2.5 ± 0.5	-	10
A × mix (M, L)	1.1 ± 0.5	-	10
A × mix (T, O)	6.3 ± 2.5	-	19
A × mix (T, L)	3.5 ± 1.5	-	14
A × mix (H, L)	0.8 ± 0.5	-	4
A × mix (P, O)	0	-	0
A × mix (P, L)	0	-	0
A × P	0	4.7 ± 2.5	42
A × mix (Con, O)	0	-	0
A × mix (Con, L)	0	-	0
A × Con	0	0.1 ± 0.1	5

*A – Asiatic hybrids, T- Trumpet and Aurelian hybrids, O - Oriental hybrids, C – *L. candidum*, M – *L. monadelphum*, R – *L. regale*, H – *L. henryi*, L – *L. longiflorum*, P – *L. pumilum*, Con – *L. concolor*

like female species *L. monadelphum.* Similar crossing combinations *L. regale* × Asiatic hybrid were fertile but set les seeds per capsule than in cases when *L. regale* was cross-pollinated by pollen of others clones of *L. regale*. Progeny in combinations *L. regale* × Asiatic hybrid look like *L. regale.* This indicated that *L. monadelphum* and *L. regale* give apomictic offspring. Reciprocal crosses (Asiatic hybrid × *L. monadelphum*, Asiatic hybrid × *L.* regale) by stigma pollination show very low fertility (**Table 1**) which depends on the particular Asiatic female genotype used. After pollination 14 different Asiatic hybrid females by *L. monadelphum* only cv. 'Vostok 2' and seedling 0-49-1 possessed very low fertility and produced 1 and 2 viable embryos, respectively. Others 12 crossing combinations were totally infertile. When the same Asiatic hybrid females were pollinated by *L. regale* only combination cv. 'Lollypop' × *L. regale* gives 2 germinated embryos. Offspring were not received in both directions of reciprocal crosses performed with *L. candidum* and Asiatic hybrid. Any progeny were obtained when native styles of Asiatic hybrid females were pollinated by *L. longiflorum*, *L. henryi*, *L. pumilum*, *L. concolor* and cultivars from O and T group.

Overcoming incongruity between Asiatic hybrid females and phylogenetically distant males

Different methods are used to overcome pre-fertilization barriers; applying cut-style, grafted style, placental pollination and *in vitro* ovule pollination methods (Van Tuyl 1991; Chi 2000). The cut-style pollination method is widely used to perform recalcitrant interspecific crosses in genus *Lilium* (Fukai and Tsuji 2004; Lim *et al*. 2008; Wang *et al*. 2009; Hamid and Kim 2010). We performed cut-style pollination to cross Asiatic hybrid females with incongruous species and cultivars from phylogenetically separated groups and to cross *L. candidum* with Asiatic hybrids and *L. longiflorum* (**Table 1**). However, progeny was obtained only in cases when cut style Asiatic hybrid females were pollinated by *L. pumilum* and *L. concolor*. Both species possess a short style and shorter pollen tube than pollen tubes of Asiatic hybrid. It tallies with the fact that pollen tube cannot penetrate the long distance and fails to reach the ovules if a seed parent has a longer style. Therefore a long style of maternal partner is important barrier preventing to perform successful crosses with *L. pumilum* or *L. concolor*. Even though, the cut style pollination allowed to obtain progeny in combinations Asiatic hybrid x *L. pumilum* and Asiatic hybrid x *L. concolor* the rate of successful seed set in both crosses was different. The fertility in crosses Asiatic hybrid x *L. pumilum* was higher than in Asiatic hybrid × *L. concolor*. Even if difference in lengths of female style and length of pollen tube are important pre-fertilization barrier in crosses Asiatic hybrid x *L. pumilum* and Asiatic hybrid × *L. concolor*, it appears likely that in crosses Asiatic hybrid × *L. concolor* an extra pre-fertilization barrier exists. It may be attributed to the receptiveness of ovule and the potential of pollen tubes for penetrating micropyles (Fukai *et al*. 2002; Fukai and Tsuji 2004).

Despite that it was impossible to receive progeny from Asiatic hybrid females after pollination with other phylogenetically distant species and cultivars, the-cut style method allowed to overcome pre-fertilization barriers when females from other lily groups were used in combinations: T x Asiatic hybrid, *L. longiflorum* × *L. candidum* and *L. longiflorum* × *L. regale* (our personal unpublished data). Chi (2000) reported that when using cut-style pollination, the generative cell divides later into two sperms than pollen tube penetration of the micropyle and that such difference in time maybe the cause of decreased efficiency in fertilization. It means that to perform incongruous crosses more efficient it is necessary to test what length of cut style is optimal for each crossing combination (Fukai *et al*. 2001, 2002; Fukai and Tsuji 2004).

Applying incompatible viable pollen in mixtures with inhibited or viable compatible pollen is known as the "mentor pollen method. However, this method maybe not always successful (Van Tuyl *et al*. 1982; Van Creij *et al*. 2000). Apparently, artificially inhibited compatible pollen in mixtures with incongruous not always stimulate fertilization (Guries 1978). Viable compatible pollen added in mixtures rivals incongruous pollen in fertilization and decreases the possibility to obtain interspecific hybrids (Song *et al*. 2002). As alternative for the "mentor pollen" method the "mixed incongruous" pollen method can be applied. Stigma pollination of native styles by mixed incongruous pollen allowed us to increase fertility in 7 different crossing combinations with Asiatic hybrid females and in one crossing combination in which *L. candidum* females were pollinated by mixed pollen of Asiatic hybrid cv. 'Red Carpet' and *L. longiflorum* (**Table 1**). However, crossing combinations where Asiatic hybrids were pollinated by pollen of *L. concolor* and *L. pumilum* in mixtures with pollen of *L. longiflorum* or OH were completely infertile. It allows speculating that incongruous pollen maybe stimulates each other by mechanism which allows overcome signals of pistil which restrict growing of pollen tubes but not by changing of tube length. To answer the question if such a stimulation is mutual or pollen of one species only donate way for growing of pollen tube of other species, special assays must be done. Identification of progeny derived from mixed pollination should add more clearness about this phenomenon.

Screening and characteristic of Asiatic hybrid × *L. candidum* and Asiatic hybrid × *L. regale* hybrids

The Asiatic hybrid maternal plants (0-42-1, 0-22-1 and 0-22-13) and the species *L. regale* and *L. monadelphum* share common isozyme banding patterns of superoxide dismutase (SOD). However, among three SOD isoforms of *L. candidum* two were detected as distinctly different. The pattern of these isoforms were used to screen Asiatic hybrid × *L. candidum* hybrids resulted after pollination Asiatic hybrid females 0-42-1, 0-22-1 and 0-22-13 by mixed pollen of species *L. regale*, *L. monadelphum* and *L. candidum* or by mixed pollen of *L. regale* and *L. candidum* (**Fig. 1**). As can be seen in **Fig. 1** the offspring selected as Asiatic hybrid × *L. candidum* inherited the three banded SOD pattern – two iso-

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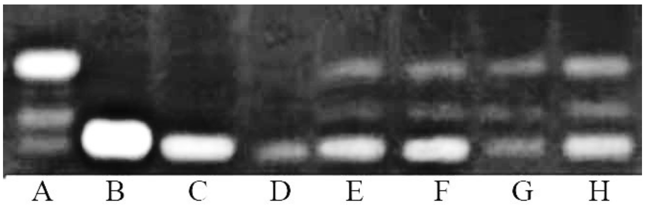

Fig. 1 Isozyme patterns for superoxide dismutase (SOD) in parental forms and hybrids. (**A**) *L. candidum*, (**B**) *L. monadelphum*, (**C**) *L. regale*, (**D**) Asiatic hybrid female 0-42-1, (**E – H**) progeny resulted from 0-42-1 pollinated by mixed pollen of *L. candidum*, *L. monadelphum* and *L. regale*.

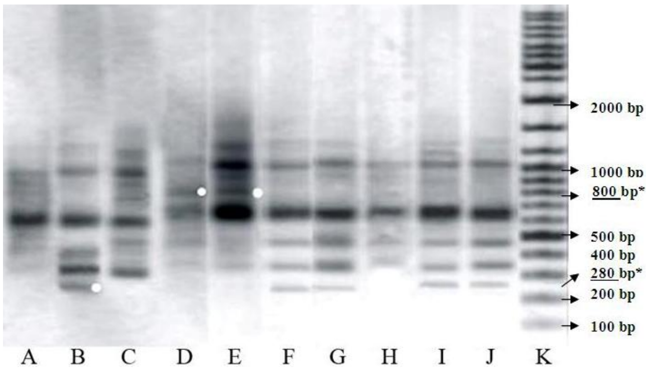

Fig. 2 Pattern of male specific RAPD markers amplified by primer Y29. Spots indicate species specific bands. (**A**) Asiatic hybrid female 0-42-1, (**B**) *L. candidum*, (**C**) *L. monadelphum*, (**D**) *L. regale*, (**E – J**) progeny resulted from 0-42-1 female pollinated by mixed pollen of *L. candidum*, *L. monadelphum* and *L. regale*, (**K**) size marker (GeneRuler™, DNA ladder Mix Nr. SM1173).

forms specific only to the paternal crossing parent *L. candidum* and one common to the maternal Asiatic hybrid and to *L. candidum*. Among 36 tested offspring resulted after pollination Asiatic hybrid with mixed pollen of *L. candidum*, *L. monadelphum*, *L. regale* 19 inherited SOD pattern of *L. candidum*. The progeny which did not inherited SOD isoforms characteristic to *L. candidum* were separated as progeny with random paternity. This group should be consist of hybrids Asiatic hybrid × *L. regale* and Asiatic hybrid × *L. monadelphum* as well, as be resulted by apomixes. The RAPD analysis allowed select in this group hybrids Asiatic hybrid × *L. regale*.

RAPD analysis provided additional evidence for identification of AC (Asiatic hybrid × *L. candidum*) hybrids screened by inheritance of SOD pattern and allowed perform the first selection of AR (Asiatic hybrid × *L. regale*) hybrid offspring that resulted from pollination Asiatic hybrid by mixed pollen. Among three primers (Y29, Y35 and Y37) tested only primer Y29 generated informative polymorphic patterns of PCR products specific to two of the paternal species *L. candidum* and *L. regale* used in the mixes. None of the primers did allowed screening for progeny of *L. monadelphum*. The 280 bp fragment resulted by amplification of *L. candidum* DNA with primer Y29 was chosen as informative marker to confirm paternity of *L. candidum* (**Fig. 2**). Since a 280 bp fragment was characteristic only for *L .candidum* but neither for maternal Asiatic hybrid nor for other paternal species *L. monadelphum* and *L. regale* it was used as important criterion to select AC (Asiatic hybrid × *L. candidum*) hybrids among progeny resulted after pollination of Asiatic hybrid with mixed pollen. All hybrids which showed the SOD pattern inherited from *L. candidum* also inherited the 280 bp fraction of DNA specific for *L. candidum.* A 800 bp fragment of DNA amplified by primer Y29 was characteristic only for *L. regale*. Inheritance of this marker in progeny with random paternity was used as only one of possible criterion to select AR hybrids (**Fig. 2**). Identification of hybrids by RAPD method not always produces pattern of amplified DNA in hybrids as exact arithmetical sum of amplified DNA of both parents. It is necessary to turn attention that recombination of DNA during meiosis can change characteristics of some randomly amplified parental fractions of DNA and in such form be presented in progeny. Therefore in this research selection of AC and AR hybrids by RAPD were based only on inheritance of a few DNA markers (280 bp DNA to select AC hybrids and 800 bp DNA to select AR). It is necessary to find more suitable species specific molecular markers for additional confirmation of selected hybrids and for discovering paternity of *L. monadelphum* and others species which were used to perform pollination with mixed pollen.

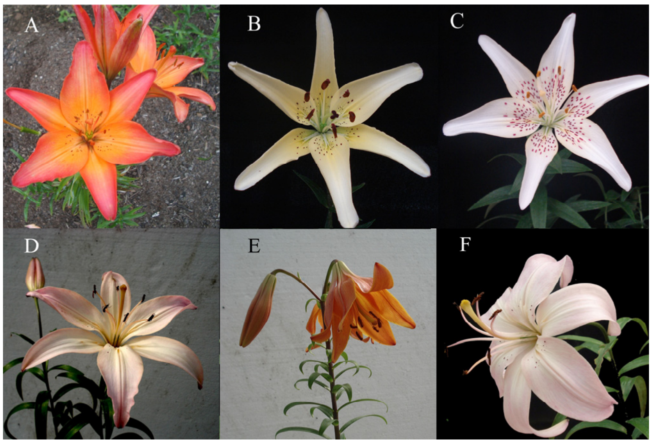

Fig. 3 Interspecific hybrids AC (Asiatic hybrid × *L. candidum*). The presented hybrids were identified as AC among progeny resulted after crossing Asiatic hybrid female 0-42-1 by mixed pollen of *L. candidum*, *L. regale* and *L. monadelphum*. (**A**) 0-42-1, (**B, C, D, E, F**) AC hybrids.

Half of progeny resulted after pollination Asiatic hybrid females with mixed pollen of *L. regale* and *L. candidum* was identified as Asiatic hybrid × *L. candidum* and the other half as Asiatic hybrid × *L. regale* hybrids. However, among offspring from families where Asiatic hybrid were pollinated by mixed incongruous pollen of *L. regale*, *L. monadelphum* and *L. candidum* only 17% of progeny consisted of Asiatic hybrid × *L. regale* hybrids and 51% were identified as Asiatic hybrid × *L. candidum* hybrids. So far it is not possible to conclude if pollen of *L. monadelphum* rival pollen of *L. regale* or not since it lacks informative molecular markers characteristic for *L. monadelphum* which should allow screening for Asiatic hybrid × *L. monadelphum* hybrids and discarding apomictic progeny.

Application of mixed incongruous pollen method allowed to perform incongruous crosses and identify 19 hybrids as AC (Asiatic hybrid × *L. candidum*) and 6 hybrids as AR (Asiatic hybrid × *L. regale*). All AC and AR hybrids phenotypically resembled Asiatic hybrid. However, among AC hybrids, plants with soft colours dominated (**Fig. 3**). The petals of some AC hybrids possess sweet smell which is much lighter than in case of *L. candidum*. The phenomenon that traits of one parent predominate in interspecific hybrids is not exceptional, e.g. crosses Asiatic hybrid × *L. pumilum* and Asiatic hybrid × *L. concolor* resulted in progenies in which many characteristics resembled the paternal species *L. pumilum* and *L. concolor*. If dominance of Asiatic hybrid traits in AC hybrids depends on crossing direction can be answered after screening and identification of reciprocal AC/CA hybrids resulted after pollination *L. candidum* with mixed pollen of *L. longiflorum* and Asiatic hybrid.

Chromosome counting showed that progeny screened as AR and AC hybrids possess 24 chromosomes. Pollen germinating test shows that almost half of AC hybrids produce viable pollen and viability of pollen in such hybrids ranged from 0.5 to 5%. Some AC hybrids possessing viable pollen were used to perform backcrosses with Asiatic hybrid females.

CONCLUSIONS

From the results obtained in this research, it can be concluded that for pollen incongruent to female pistil chance of fertilization can increase if such pollen is used in mixtures with other incongruent pollen. The Mixed pollen method allows to overcome pre-zygotic isolation barriers and to perform interspecific hybridization between distantly related species. Simultaneously, with the use of the mixed pollen method it is possible to succeed in making different interspecific crosses that are normally incongruous in a single pollination, e.g. AC and AR hybrids were obtained when Asiatic hybrid were pollinated by mixed pollen of incongruent species *L. candidum* and *L. regale*.

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