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Full Length Research Paper

Cross compatibility between *Lilium* x *fomolongi* group and *Lilium*.*brownii*

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Lilium is an ornamental plant with great worldwide commercial importance. Obtaining intra- and interspecific hybrids is a known approach to introduce new traits into the commercial groups and cultivars. However, considering the incompatibility of many intra- and interspecific crosses, resulting from pre-fertilization and post-fertilization barriers, various methods have been employed to obtain new hybrids. *L. longiflorum* is a Lilium group with great marketability, which exhibits incompatibility in many crosses. Here, we have tried to obtain interspecific *L* . × *fomolongi* × *L. brownii* and *L. longiflorum* × *L. brownii* hybrids using the combination of ovary slice and ovule culture. In the study, we obtained some interspecific hybrids in *Eorayon 2ho* × *B (7),* and *Augusta* × *B (7),* as well as *Augusta* × *KHR, Afjw* × *KDD,* and *Augusta* × *KDD* crosses. To the best of our knowledge, this is the first reported successful study obtaining *L.* x*fomolongi* × *L. brownii* hybrids. Evaluating the morphological characteristics of the hybrids obtained, as well as studying the traits introduced from parents, can be topics for future studies

Key words: *Lilium*, ovule culture, hybrid, ovary slice culture, embryo rescue, SSR.

INTRODUCTION

Lilium is of great importance in ornamental plant market and is widely used throughout the world. Different groups and cultivars of Lilium are spread worldwide with a wide range of physical characteristics, adaptation and susceptibility to different climates and pests (Wang et al., 2009). Over the past few years, the importance of lily has increased enormously, especially in The Netherland (Kapoor et al., 2009). Considering the high acceptance of the plant as well as its great market throughout the world, many breeding programs have been carried out on different Lilium cultivars and groups (Lim et al., 2008). However, there are some difficulties in this respect. A good instance of the issue is *L. longiflorum*. Great marketability of *L. longiflorum* is due to its trumpet–shaped flower with distinctive fragrance, and its easy year-round cultivation (Mc Rae, 1998). Thus, many breeding programs have so far been carried out on intra- and inter-specific hybrids (Kanoh et al., 1988; Sheiichi and Keita, 2004; van Tuyl et al., 1986; van Tuyl and van Dien, 1991; Wang et al., 2009). However, *L. longiflorum* has demonstrated interspecific incompatibility with many groups and cultivars, due to inhibition of interspecific pollen growth or underdevelopment of the embryo.

Lilium brownii mainly originated from China (Long and Zhang, 1998) . When it was introduced to Europe in the 18th century, it was one of the most expensive and exquisite groups of lilium, considering its good flower fragrance and nice appearance. The plant also has some application as food and medicine in Korea and China. Moreover, the plant demonstrates strong cold resistance, virus resistance, as well as strong drought resistance (Long et al., 1999). In addition to its ornamental uses, in recent years *Lilium brownii* has attracted the attention of researchers as a candidate for different medical applications (Ehrman et al., 2010; Lin et al., 2003; Wang and Bun, 2002; Zeng et al., 2008). However, it is susceptible to mite and soil insects (Long et al., 1999).

Considering the desirable characteristics of *L.* x *fomolongi*, it has been an appropriate candidate for interspecific hybrids. However, as was aforementioned, in spite of the large number of attempts to produce interspecific hybrids of *L.* x *fomolongi*, the plant shows

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Table 1. Lily genotypes used for hybridization.

Group	Cultivar
L xfomolongi	Augusta'Eyorayon1ho'Eyorayon2ho'Eyorayon3ho'Lorina,'Afjw,'Raizon Herald' (Jinsan× White American*)*
L. brownii	Kyodongdo (KDD)' Jeongpek y y,'B (3)' Kyoharo (KHR)' Wonsando, Sanvonsan, B (7)' Hoachon, Yajo'Yongjonam mion
L. longiflorum	White American' Jeorjia

incompatibility in many crosses. To the best of our knowledge, and considering the literature in this regard, no study so far has been carried out to evaluate the possibility of production of interspecific hybrids of L.x fomolongi and L. brownii. Our previous attempts to produce L.x fomolongi and L. brownii hybrids, in which L.xfomolongi, contributed to the cross as the male parent failed to produce fruit set. Thus, the present study is the first of its kind in this regard to address the issue of producing L.xfomolongi and L. brownii hybrids with L.x fomolongi, as the female parent. In this study, we have tried to produce some interspecific hybrids of L. longiflorum and L. xfomolongi, with some cultivars of L. brownii groups. However, as was mentioned, the main focus of the study is to obtain hybrids of L.x fomolongi, × L. brownii. As the first step, we tested the germination potential of the cultivars' pollens used in the study, and then performed some interspecific crosses using controlled hand-pollination to evaluate pollen tube growth. As the focus of the study was to obtain L.x fomolongi, × L. brownii hybrids, we performed crosses between the two groups using cut-style pollination method to obtain hybrids. Consequently, pollinated ovaries were used for ovary slice culture and ovule culture. These methods were employed to produce a higher number of plantlets (if at all viable), as well as to overcome the pollen tube development barriers.

MATERIALS AND METHODS

Plant material

For the purpose of this study, 8 *L.xfomolongi*, cultivars, 10 *L. brownii* cultivars, and 2 cultivars from *L. longiflorum* group were involved in the crosses (Table 1). The bulbs were obtained from the Flower Breeding Research Institute at Kangwon National University, Korea. The bulbs were stored at -2°C, and were transferred to greenhouse and planted in pots from January to early March 2009. Greenhouse temperature was maintained at 22 to 25°C during the day and at 15°C at the night. The genotypes used for hybridization are shown in Table 1. The chemical (Ethanol, Acetic acid, NaOH, NaCl) from (Merck, p.a.), was used without further purification.

Pollen viability test

Before pollination, pollen viability was checked on pollen collected from all genotypes used in the hybridization. The pollen was put on Petri dishes in a culture medium (20 g L^{-1} sucrose, 10 mg L^{-1} boric acid, 7 g L^{-1} agar) and cultivated at 25°C. Pollen germination was recorded after 1, 2, 4, 6 and 8 h for all genotypes. The viability of pollen was expressed as percentages. In hybridizations, only genotypes with a pollen viability 5% and higher were used (Table 2).

Pollination methods

The pollen from the healthy and mature cultivars was collected in the morning and used for pollination. We employed two pollination methods, normal pollination and cut-style pollination. After performing pollen viability test, pollen tube growth in the styles of different crosses was specified following stigma pollination that is, mounting the pollen of the desired cultivar on the intact stigma. 12, 24, 48, 72 and 96 h after pollination, the ovaries were separated and fixed in 70% ethanol. After styles were separated from the ovary solution, the samples were kept in 70% ethanol- acetic acid (3: 1) for 24 h. Then, they were washed three times with distilled water and kept in NaOH (2N) for six hours, and again washed three times with distilled water to be later kept in aniline blue solution for 24 h.

In the next step, styles were taken out of the solution and were flattened with a cutter on the slide, and then pollen tube growth through the pistil was examined by fluorescent microscope, and expressed as the percentage of pistil length passed by the pollen tube.

In order to carry out cut-style pollination, *L.xfomolongi* , and *L. longiflorum* flowers were emasculated before anthesis and then the stigmas were covered by aluminum foil. As the exudates of stigmas appeared, the styles of flowers were cut 1 cm above the ovaries with a razor blade and the pollens of respective cultivars were administered to the cut surface for hybridization. The styles were then covered with aluminum foil again. Hybridizations were conducted from April to early May in the greenhouse.

Preparation of ovaries

Immature ovaries were collected 35 to 70 days after pollination. The ovaries from the healthy explants were sterilized in 70% ethanol for one minute followed by rinsing twice with sterile distilled water and subsequently soaked in 1% sodium hypochlorite for 20 min, followed by three times rinsing with sterilized distilled water.

Ovary slice culture

More than 40 days after pollination (DAP), ovaries of some crosses were picked and the swollen parts of the ovaries were sliced into disks 2 mm thick. About 4 to 5 disks were obtained from one ovary and one disk contained 30 ovules on average. A modified MS agar medium (pH 6.3, 8% sucrose) was used as the test medium. The ovary disks were inoculated on the test medium, and then cultured

Table 2. Change of Pollen viability test of different group of Lilies after 8 hour after culture in *invitro*.

Lilium groups	Cultivar	NEP*	8 h after culture
L. x *fomolongi*	*Augusta*	256	32.7 ± 0.62^{f}
	Eyorayon 1ho	235	89.6 ± 1.23^{ab}
	Eyorayon 2ho	237	93.7 ± 0.94^{a}
	Eyorayon 3ho	243	85.2 ± 2.24^{c}
	Afjw	229	90.2 ± 1.60^{ab}
	Lorina	278	80.2 ± 1.84^{d}
	R.Herald	248	89.1 ± 3.87^{bc}
L. brownii	*Kyodongdo*	219	85.3 ± 2.36^{c}
	Jongpek y y	196	21.9 ± 1.63^{g}
	B (3)	182	7.9 ± 1.63^{h}
L. longiflorum	*White American*	377	38.7 ± 1.41^{e}
	Jeorjia	268	25.1 ± 1.93^{g}

* NEP: Number of evaluated pollen.*The data represent the mean number of ovules germinated per explants ± SD of three independent experiments. Value within a column followed by different letters is significantly different at the 0.05 probability level using Duncan's multiple test ($P < 0.05$).

at a temperature 25 ± 1°C under continuous illumination of 1500 lux.

Ovule culture

The ovules containing embryos were excised aseptically from the protruding points on the ovaries, and were subsequently placed in the media; containing full-strength basal medium and sucrose (6%) for ovule germination. All media were adjusted at pH 5.8. The ovules were inoculating in MS (Murashige and Skoog) medium (Murashige and Skoog, 1962) supplemented with auxin (NAA). Ovule cultures were kept in 9×4 cm plastic culture dishes at 24°C, with 16 h photoperiods, and after 36 to 69 days, number of ovules germinated and also the numbers of seedlings from germinated ovules were recorded.

Growth of hybrids

The hybrids obtained were acclimatized until they become sufficiently hardy to survive in uncontrolled field conditions. By then, they were transferred to soil, to be used for back-crossing and producing seeds for F_2 further studies.

RESULTS AND DISCUSSION

The groups and cultivars used in this study are shown in Table 1 and Figures 1 to 3. The results of pollen germination on culture medium one, two, four, six, and eight hours after being cultured are provided in Table 2. As it is clear, the evaluated cultivars were different in terms of pollen germination, such that in *L.* x *fomolongi*, cultivars Eyorayon 2ho, Lorina, Eyorayon 1ho, Eyorayon 3ho showed the best pollen tube growth on the media, while Kyodongdo (KDD) in *L. brownii* group had the longest pollen tube length after eight hours. It should be noted that if we consider the pollen tube length of Lilium groups studied, but eight hours after pollen culture, the three groups of Lilium were not significantly different in this regard (Figure 4).

Then, we carried out some crosses between the cultivars of *L. longiflorum* and *L.* x *fomolongi*, with *L.* brownii by controlled simple stigma pollination such that *L.* x *fomolongi*, cultivars were pollen recipient (Figures 5 and 6). The rate of pollen tube growth was different in various crosses. 96 h after pollination, among crosses in which Augusta cultivar involved Augusta × KDD, Augusta × Sanvonsan, and Augusta × Jongpek y y crosses reached the base of style. Moreover, Among *L.* x *fomolongi*, × L. brownii crosses in which cultivar involved, the best results was observed in (Jinsan × W.A.) × KDD (92.8%) and Eyorayon 2ho × KDD (79%) were the best results obtained in *L.* x *fomolongi*, × L. brownii crosses, (Jinsan × W.A.) and Eyorayon 2ho involved, respectively.

Also in self crosses of Augusta × self and Eorayon 3ho × self pollen tube reached the base of style after 96 h. The highest pollen tube growth in *L. longiflorum* × *L. brownii* crosses 96 h after performing the cross-pollinations were observed in White American yajo and White American x hoachon in which pollen tube reached the base of styles.

As the focus of the study was *L.* x *fomolongi* × *L. brownii* hybrids, we carried out 17 cross-pollinations between the two groups using cut-style pollination method, containing most top rank results of stigma pollination, and consequently performed ovary slice or ovule culture. As can be observed, in Eorayon 2ho× B(7)

Table 3. Seedling of interspecific hybridization between *L.* x *fomolongi* and L.*brownii*

Cross	No of flowers	No of fruit set	Percent of fruit set (%)	No of fruits culture	DAP	No of disks	No of ovule culture	Seedling
Eorayon1 ho X B(KDD)	6	2	33.3	2	36	12	-	27
Eorayon2 ho X B(KDD)	32	15	46.8	4	48-54	19	-	4
Herald X B(KDD)	15	4	26.6	4	39-43	16	-	8
Eorayon3 ho X B(KDD)	5	1	20	1	41	3	-	-
Augusta X B(KDD)	24	16	66.6	2	63	-	29	-
W. American X B(KDD)	13	0	0	-	-	-	-	-
(JinsanX WA) X B(KDD)	22	5	22.7	4	40-53	14	-	-
Afjw X B(KDD)	12	9	75	3	36-43	14	-	-
Lorina X B(KDD)	15	7	46.6	1	54	-	10	-
Eorayon2 ho X B(7)	4	4	100	2	45	13	-	7
Augusta X B(KHR)	19	17	89.5	9	58	-	104	14
Augusta X B(7)	34	34	100	1	52	5	-	3
W. American X B (JPYY)	4	0	0	0	-	-	-	-
Herald X B(3)	7	2	28.6	2	43	14	-	13
(Jinsan X WA) XB(7)	8	2	25	1	44	19	-	3
Eorayon 2 ho X B(KHR)	5	2	40	1	42	5	-	1
Jeorjia x B(KDD)	15	0	0	-	-	-	-	-

and, Augusta × B (7) crosses, all flowers produced fruit, while in White American × KDD, White American × Jeongpek y y, and KDD × Eyorayon 1ho crosses, none of the flowers produced fruit. Moreover, 89.5, 75, 71.4 and 66% of flowers in Augusta × B (KHR), Afjw ×B(KDD), and Augusta × B(KDD) crosses transformed into fruit, respectively.(Table 3)

Regarding the great worldwide economical importance of the L. longiflorum, various studies have been carried out to obtain interspecific hybrids of L. longiflorum with modified characteristics so far (Asher and Peloquin, 1968; Kanoh et al., 1988; Sheiichi and Keita, 2004; van Tuyl et al., 1986; van Tuyl and van Dien, 1991; Wang et al., 2009). Considering the interesting characteristics of L. brownii, that is, its good flower fragrance and nice appearance, as well as strong cold resistance, virus resistance, and strong drought resistance (Long et al., 1999), it is a potential candidate to obtain interspecific hybrids with L. longiflorum. However, many Lilium cultivars are incompatible in intra- and interspecific (van Tuyl and van Dien, 1991). To best of our knowledge and regarding the literature in this respect, this study is the first successful report to address the issue of producing hybrids of L.xfomolongi × L. brownii in which pollen of L. brownii employed.

Since the interspecific incompatibility of lilium results from inhibition of interspecific pollen growth or underdevelopment of the embryo from pre-fertilization and post-fertilization barriers (Kanoh et al., 1988; Prosevicius and Strikulyte, 2004), we used cut-style pollination, and ovary and ovule culture methods to overcome these barriers.

Moreover, we employed two other Lilium groups in interspecific crosses. Firstly, we tested the pollen germination of cultivars used in this study on culture medium. As it was shown in Table 2, among the L. brownii cultivars, KDD showed the best pollen tube germination on culture medium after eight hours. Thus, it can potentially show better results in stigma pollination crosses. The findings of stigma pollination, provided in Table 3, show that in crosses carried out between different cultivars of L.xfomolongi and L. brownii crosses, KDD pollen had the best results in various crosses Augusta and (Jinsan × W.A.) involved.

This is suggestive that in spite of the existence of some pre-fertilization factors, the crosses in which KDD participate as the pollen donors are potentially able to produce interspecific hybrids. To overcome the pre-fertilization barriers, different methods have been used so far, including applying a mixture of pollens from several species, cut-style, grafted style, placenta pollination and *in vitro* ovule pollination, each of which has its particular advantages and shortcomings (Asano and Myodo, 1977; Chi, 2000; Prosevicius and Strikulyte, 2004; van Tuyl and van Dien, 1991). Also, different methods have been employed to circumvent post-fertilization barriers, including embryo rescue, ovary slicing and ovule culture (Chi, 2002; Prosevicius and Strikulyte, 2004). It should be noted that among the different methods employed to overcome post-fertilization barriers, ovary culture produces more fruits compared embryo rescue method (Sheiichi and Keita, 2004), hence, in spite of being

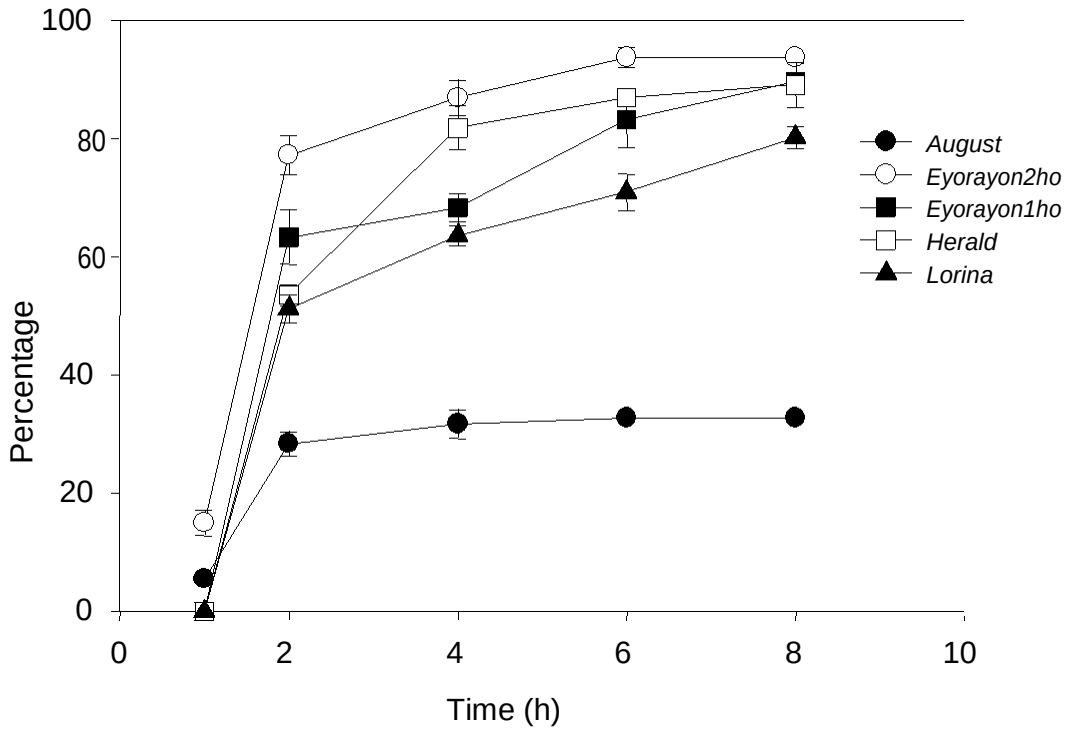

Figure 1. Change of pollen germination of different *L.*× *fomolongi* lines in *in vitro* (Bars mean standard deviation).

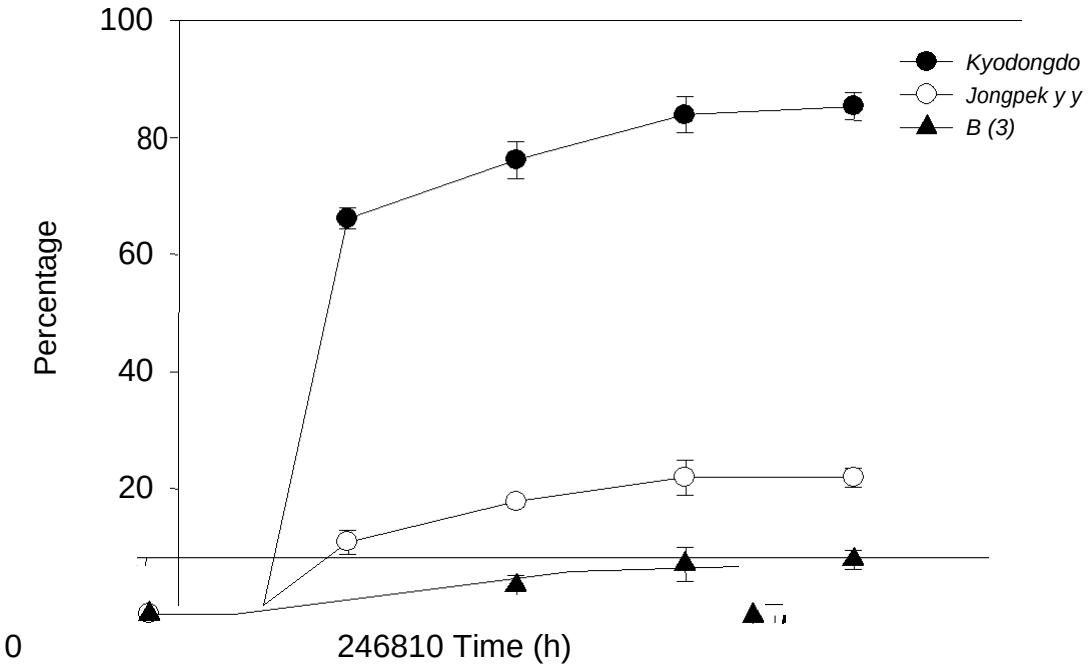

Figure 2. Different of pollen germinabilities in *in vitro* of *L. brownii* lines (Bars means standard deviation).

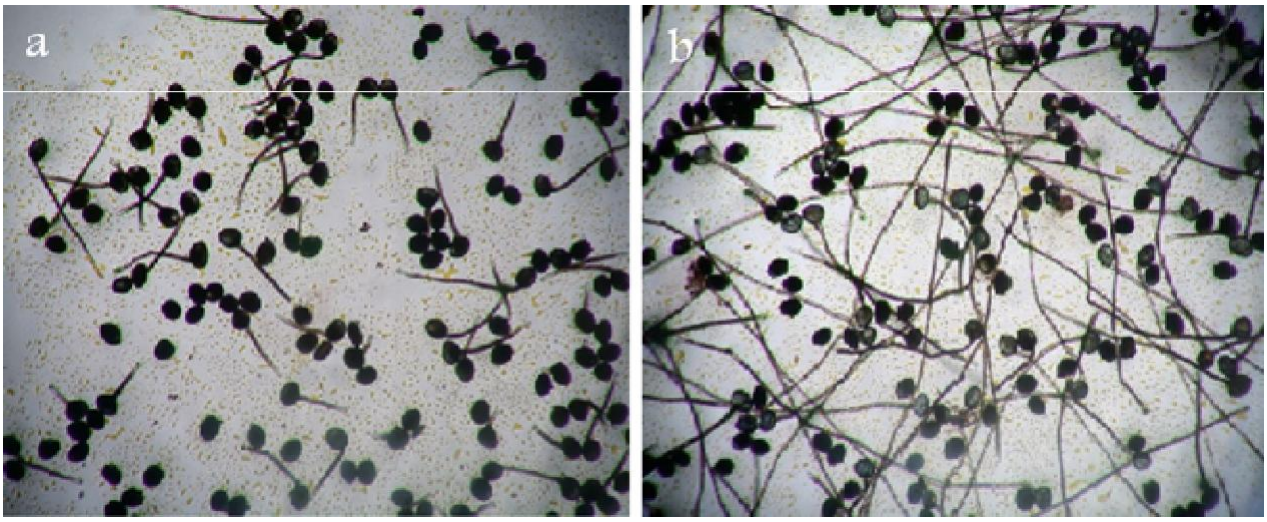

Figure 3. Pollen germination test of Eyorayon 2ho *in vitro*, (a) after 2 h, and (b) after 4 h.

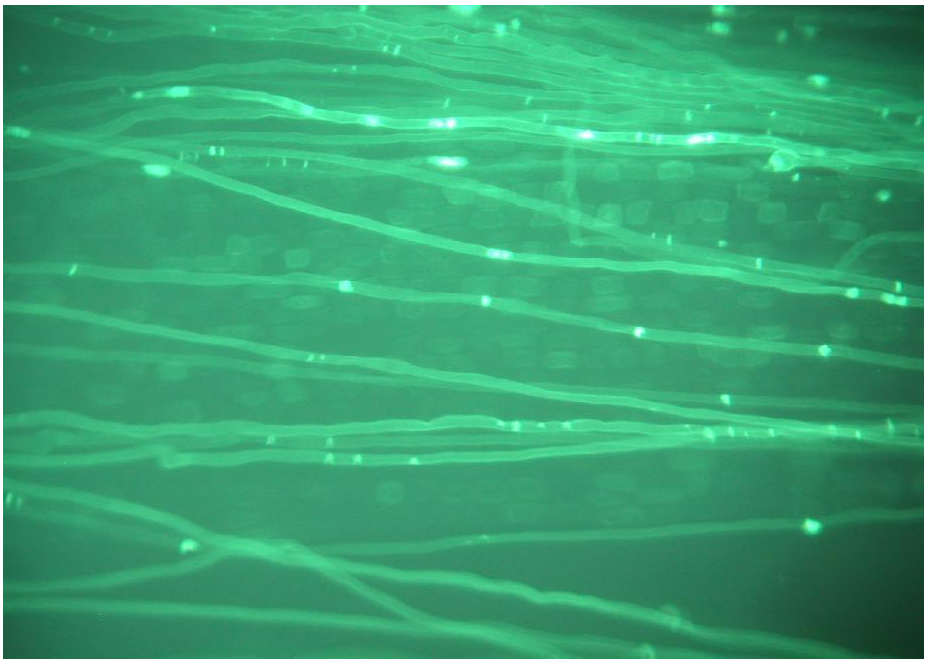

Figure 4. Pollen tube in the cross between *L*. ×*fomolongi* × *L.brownii (*Augusta × KDD).

laborious, we used ovary culture method to achieve this goal.

In this study, we adopted cut-style pollination to bypass pre-fertilization barriers. Moreover, to overcome the post-fertilization barriers as well as increasing the number of obtained plantlets (if at all viable), after pollination, ovary slice and ovule culture methods were employed, as mentioned in methodology. Since the focus of the study was *L. longiflorum* × *L. brownii* hybrids, we used cut-style pollination, and ovary and ovule culture for these crosses. Considering the high potentiality of KDD to produce interspecific hybrids, we performed crosses of KDD and all the L.xfomolongi cultivars employed in the study.

As it can be observed, in cut-style pollination, 100% of flowers in *Eorayon 2ho* × B (7), and *Augusta* × B (7) crosses produced fruit set. Moreover, 89.5, 75 and 66% of flowers in Augusta × KHR, Afjw × KDD and *Augusta* × *KDD* crosses transformed into fruit set, respectively.

We concluded from the stigma pollination results that KDD can be a potentially good candidate for production of interspecific hybrids of *L.* x *fomolongi* × *L. brownii*. Consistently, results obtained in cross-pollination

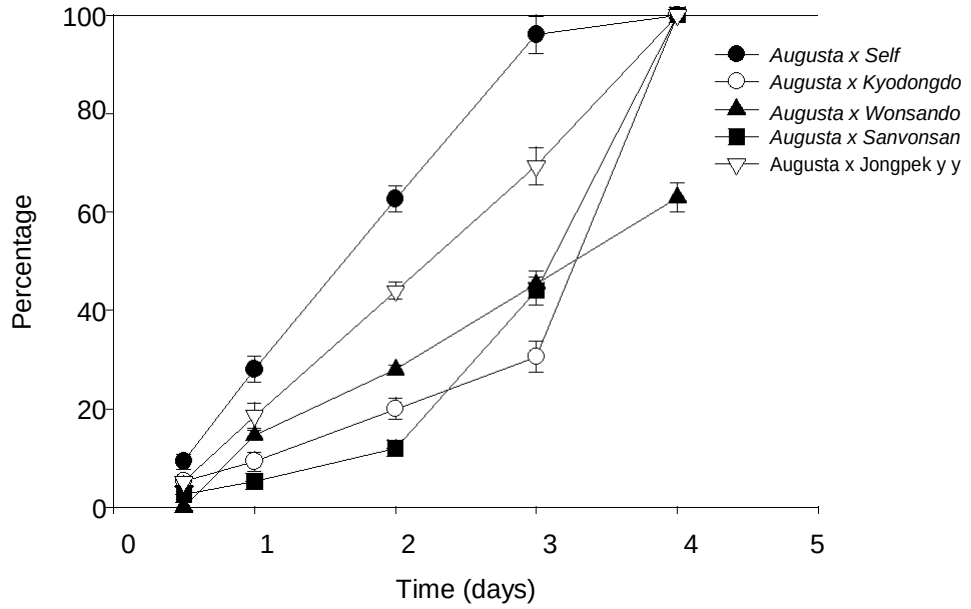

Figure 5. Change of pollen tube length in cross between Augusta × *L. brownie.*

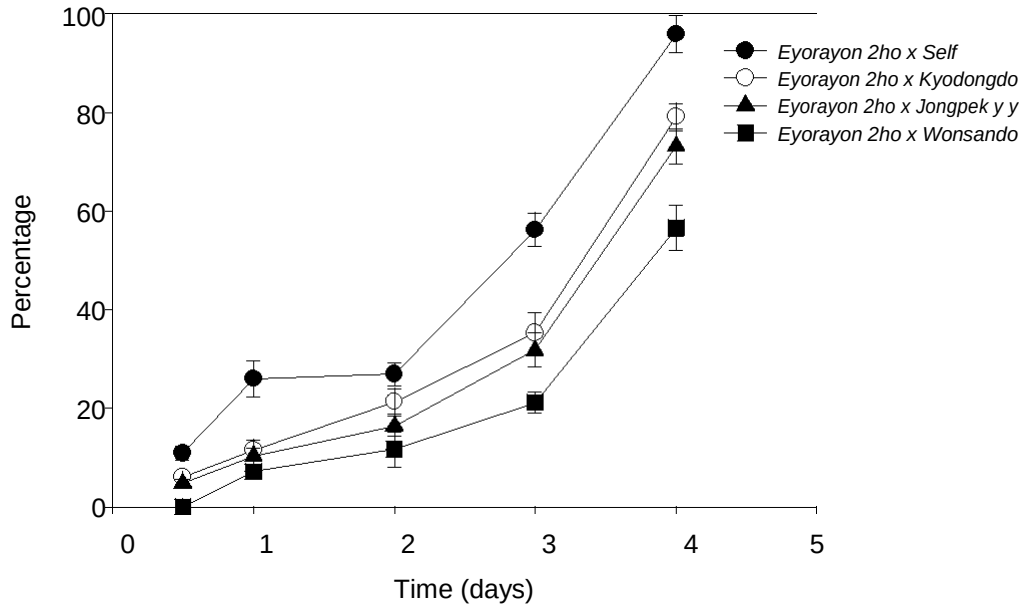

Figure 6. Change of pollen tube length in cross between *Eyorayon 2ho* × *L.brownii.*

indicated that some crosses including *Eorayon 2ho* × B (7) and, *Augusta* × B (7), as well as Augusta × KHR, Lorina × KDD and Augusta × KDD are able to produce seedling hybrids.

Analysis of F_1 seedling by SSR markers

To confirm the hybridity of obtained plants, we analyzed them using SSR marker .This study selected a number of SSR primer pairs for the identification of Lily hybrid. The polymorphisms observed between the parents are used as markers for hybrid identification. Comparing the SSR markers banding pattern of parents with respective hybrids, genuine hybrids were confirmed (Figure 7). Of all the primers used in this study, L 37, L50, L61 and L67 produced highly polymorphic patterns in 4 putative hybrids with complementary banding pattern of both parents. The SSR marker, L37 and L50 used to differentiate hybrid and parents lines obtained from cross

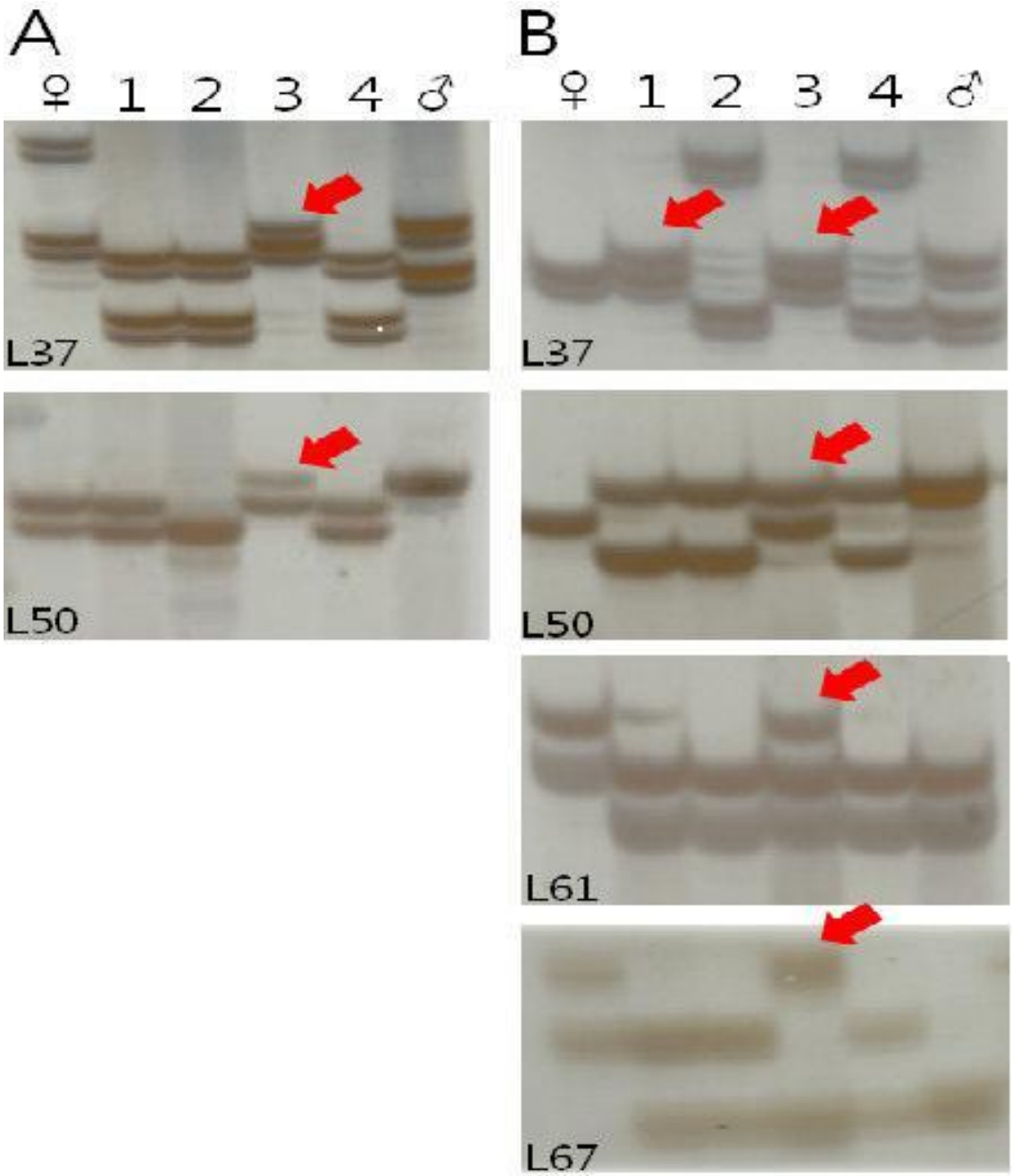

Figure 7. Hybrid confirmation using SSR markers. (A) Eyorayeon-1 ( ) x *L. brownii* (*KDD*) ( ), (B) Eyorayeon-2 ( ) x *L. brownii* (*KDD*) ( ). The arrows indicate heterozygotes having both parental alleles. Among the 4 putative hybrids in A and B, the hybrids in the third lanes were confirmed to have both parental alleles.

between Eyorayon 1ho ( ) x *L. brownii (KDD)* ( ) (Figure 7A). SSR marker L37, L50, L61 and L67 were used to differentiate hybrid and parental lines obtained from cross between Eyorayon 2ho ( ) x *L. brownii* (*KDD*) ( ) (Figure 7B). Variation in marker from the parents to hybrids may have originated due to recombination, deletion, mutation or random segregation of the chromosomes at meiosis during the process of hybrid formation (William et al., 1990; Tzeng et al., 2009).

These are the first successful interspecific hybrids of *L. longiflorum* × *L. brownii* and *L.*x *fomolongi* × *L. brownii* reported to be obtained until now.

The main of goal of this study was to obtain viable interspecific hybrids between *L.*x *fomolongi* and *L. longiflorum* with *L. brownii*. However, as the attempt was the first of its kind as employed *L. brownii* in production of interspecific hybrids with *L. longiflorum* and *L.*x*fomolongi* (*L. brownii* as the male parent); (Figure 8) it was the focus of our

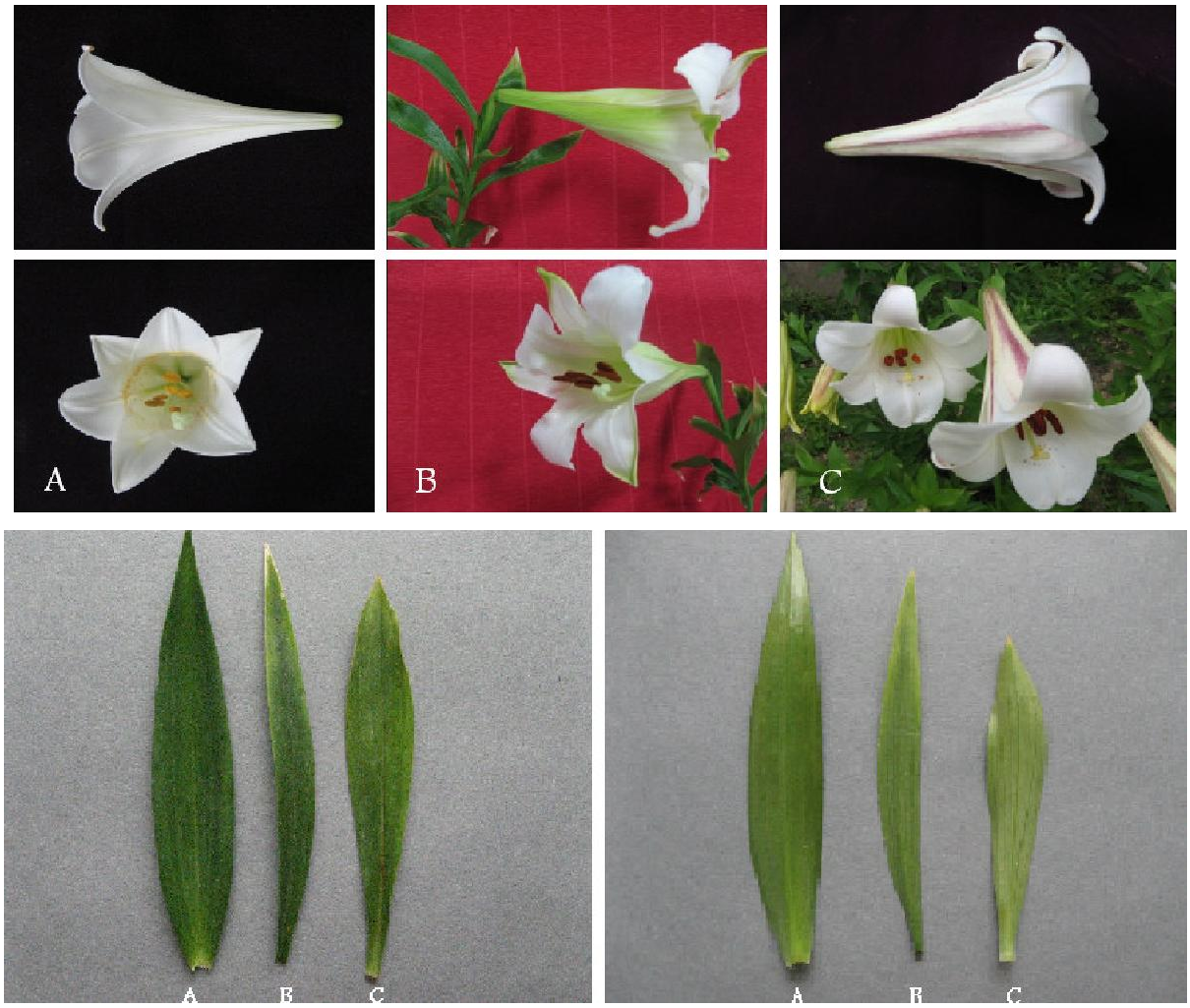

Figure 8. Plants of parental species and hybrid (A) *L. xfomolongi,* (B*) L. fomolongi x L. brownii (*Eyorayon 2ho × KDD)*,* and (C) *L. brownii* (KDD).

study, and finally the hybridity of the obtained plants were analyzed using SSR method. We have accomplished the goal of obtaining such interspecific hybrids in this study. The next step would be analyzing the characteristic of obtained hybrids to examine the new traits incorporate into them, as well as their new physical appearance. As morphological characteristics and their inheritance can be best evaluated from F2 on, this can be the subject of further studies.

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