

胚培養を用いた不稔種Lycoris incarnataを交配親とする種間雑種の作出

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Production of Interspecific Hybrids Between *Lycoris incarnata* and Four Other *Lycoris* Species through Embryo Culture

Biao Ma¹, Isao Tatumoto^{1*}, Noriyoshi Nakamura² and Hisato Kunitake³

¹ Graduate School of Agriculture and Biological Science, Osaka Prefecture University, Sakai, Osaka 599–8531

² Saga Prefecture Agricultural Research Center, Saga, Saga 840–2205

³ School of Agriculture, Kyushu Tokai University, Kumamoto 869–1404

Summary

Interspecific crosses were made between a sterile species (*L. incarnata*) and four fertile species (*L. sanguinea*, *L. sprengeri*, *L. radiata* var. *pumila* (*L. pumila*, hereafter), and *L. aurea*) to breed varieties with new characters. Embryos, excised from ovules 20–30 days after pollination, were cultured on a modified MS medium containing 500 mg · liter⁻¹ casein hydrolysate (Ma et al., 2001). Interspecific crosses using *L. incarnata* as a female parent yielded 26 hybrid plants, whereas that of *L. sanguinea* × *L. incarnata* produced none. Cytological studies revealed that all hybrids were triploid, which would have originated from the fertilization of an unreduced 2n-egg of the female parent (*L. incarnata*) and a normal haploid gamete of the male parent. The hybridity was also confirmed by isozyme analysis in 26 hybrid plants and partly by character observation in hybrid plants of *L. incarnata* × *L. pumila*.

Key Words: embryo culture, interspecific hybridization, isozyme, *Lycoris*, triploid.

Introduction

The genus *Lycoris*, a member of the family Amaryllidaceae, contains about 20 species. Most of the species are commonly cultivated in China, Japan, and the United States (Hsu et al., 1994). Recently, the demands for cut flowers of *Lycoris* increased with diversification of flower consumption, so breeding of varieties with new flower forms and/or colors became desirable for *Lycoris* (Katsukawa et al., 2000). At present, the hybridization between *Lycoris* species is most promising for improving and diversifying certain traits.

In interspecific hybridization without any artificial treatment after pollination, the success of attaining progenies between fertile *Lycoris* species is low, ranging from 0.09 plants per floret in *L. aurea* × *L. radiata* var. *pumila* (Takemura, 1962) to 0.38 plants per floret in *L. sprengeri* × *L. chinensis* (Xu et al., 1986). Crosses between fertile and sterile *Lycoris* species as well as between sterile *Lycoris* species have never been successful (Xu et al., 1986; Lin et al., 1990).

Sterility in *Lycoris* species is attributed to karyological abnormalities so that in interspecific hybridization using sterile *Lycoris* species, although fertilization ordinarily occurs, hybrid embryos are generally weak and prone to abort due to chromosomal unbalance (Inariyama, 1951; Koyama, 1955; Isobe and

Yazawa, 1993). To prevent embryo abortions, 'Mizusashi method' of keeping the scapes in a flask of water after pollination (Tokugawa and Emoto, 1930), has often been used in *Lycoris*, and embryo rescue was achieved in few interspecific crosses using sterile species (Koyama, 1955; Katsukawa, 2000). However, the efficiencies of hybrid production with 'Mizusashi method' are very low, ranging from 0.03 plants per floret in *L. radiata* × *L. sanguinea* (Koyama, 1955) to 0.25 plants per floret in *L. radiata* × *L. aurea* (Katsukawa et al., 2000). Hence, there are still many sterile *Lycoris* species whose interspecific hybrids have not been produced. Recently, embryo, ovule and/or ovary culture techniques have successfully been applied for rescuing embryos in *Lycoris* (Isobe and Yazawa, 1993; Ma et al., 2000; Ma et al., 2001); the rates for producing hybrid plants were increased to 0.53 plants per floret in *L. radiata* × *L. sanguinea* (Ma et al., 2000). Particularly, an ovule culture method developed by Ma et al. (2001) has proved to be promising for producing interspecific hybrids with sterile *Lycoris* species.

Several traits of *L. incarnata* Comes ex Spreng. such as funnel flower form, light rose color, and strong scapes are desirable for developing varieties with these characters. However, it has been difficult to produce interspecific hybrids using *L. incarnata* because of its sterility. In this study, we made interspecific crosses between the sterile species (*L. incarnata*) and four fertile *Lycoris* species, and succeeded in obtaining progenies through an embryo culture technique. The hybridity of

those plants was confirmed by cytological studies, isozyme analysis, and partly by morphological observations.

Materials and Methods

Plant materials and pollination

Five *Lycoris* species including a sterile species (*L. incarnata*) and four fertile species [*L. sanguinea*, *L. sprengeri*, *L. radiata* var. *pumila* (*L. pumila*, hereafter), and *L. aurea*] were used as parents. The plants were grown in the fields of Osaka Prefecture University (Sakai) or Saga Prefecture Agricultural Research Center (Saga). Scapes were cut just before anthesis and kept in flasks filled with tap water according to Koyama (1959). Flowers of female parents were emasculated before anthesis and pollinated with the corresponding pollens stored in a refrigerator at -30°C (Mori et al., 1992), because the flowering time of *L. incarnata* differed from those of the other parental species. Four interspecific crosses using *L. incarnata* as the female parent (Table 1) and one reciprocal interspecific cross of *L. sanguinea* \times *L. incarnata* were carried out at Sakai in 1998, whereas the cross of *L. incarnata* \times *L. pumila* was done at Saga in 1993. Self-pollination of *L. incarnata* and *L. sanguinea* was made at Sakai in 1998 as a control.

To examine pollen germinability, a germination medium, containing 10% sucrose and 1.5% gellan gum (Wako Pure Chemical Industries, Ltd.), pH 5.8, was used. Pollen was incubated on the medium at 25°C in the dark for 5 hours, and the germination rates were recorded (Mori et al., 1992).

Embryo rescue

Ovaries were collected between 20 to 30 days after cross- or self-pollination, surface-sterilized with 70% ethanol for 60 sec, and then with 2% sodium hypochlorite for 20 min, and finally rinsed thrice with sterilized water. The well-developed ovules with dark brown color which seem to contain culturable embryos were cut out from the ovaries after interspecific pollinations using *L. incarnata* as a female parent, and embryos were excised from the ovules under a stereomicroscope. In the cross of *L. sanguinea* \times *L. incarnata* and self-pollination of *L. sanguinea* and *L. incarnata*, the ovules were cultured *in vitro*, because they were not well-developed 20–30 days after pollination. Excised embryos and/or ovules were placed on a modified MS medium containing $825\text{ mg}\cdot\text{liter}^{-1}\text{ NH}_4\text{NO}_3$, $350\text{ mg}\cdot\text{liter}^{-1}\text{ KCl}$, $880\text{ mg}\cdot\text{liter}^{-1}\text{ CaCl}_2\cdot 2\text{H}_2\text{O}$, $500\text{ mg}\cdot\text{liter}^{-1}$ casein hydrolysate (CH), $30\text{ g}\cdot\text{liter}^{-1}$ sucrose, and $2\text{ g}\cdot\text{liter}^{-1}$ gellan gum (Ma et al. 2001). The medium was adjusted to pH 5.8 before autoclaving. Cultures were incubated at $25 \pm 1^{\circ}\text{C}$ in the dark until germination, then at $25 \pm 1^{\circ}\text{C}$ under a 16-hr photoperiod. The seedlings obtained were transferred onto solid MS medium containing $60\text{ g}\cdot\text{liter}^{-1}$ sucrose and $2\text{ g}\cdot\text{liter}^{-1}$ gellan gum to

promote bulb development.

Cytological study

Root tips of parents and hybrid plants were pretreated with cold water at 0°C for 24 hr, then fixed in Farmer's fluid for 2 hr. They were hydrolyzed in 1 N HCl for 6 min at 60°C , stained with Feulgen solution for 15 min at room temperature, and squashed in 45% acetic acid. The chromosome number was counted in at least 5 metaphase plates per plant, and individual chromosomes were classified into three types: M (metacentric chromosome), A (acrocentric chromosome), and T (telocentric chromosome), according to Kurita (1987, 1988).

Isozyme analysis

To obtain further evidence of hybridity, isozyme analysis was carried out for acid phosphatase (APT) and glutamate-oxaloacetate transaminase (GOT). Leaves of hybrid plants, as well as their parents, were used for enzyme extraction. Horizontal starch-gel electrophoresis was carried out using 11% gels (Sigma). Extraction, electrophoresis and isozyme staining were performed according to Tanksley and Orton (1983).

Character observation

To verify the hybridity, two plants of *L. incarnata* \times *L. pumila* were observed for several traits according to the description of the genus *Lycoris* (Hsu et al., 1994).

Results

Production of hybrid plants

Pollen germination rates of four fertile species (*L. sanguinea*, *L. sprengeri*, *L. pumila*, and *L. aurea*) were 50–60%, whereas pollen germination rate of *L. incarnata* (sterile species) was very low (0.5–2.0%) (Table 1).

In all the cross- and self-pollinations, ovaries started to enlarge five days after pollination and developed into capsules within 20 days. Capsule formation rates were 60–90% in four interspecific crosses using *L. incarnata* as a female parent; those capsules developed into normal size at maturity. Although most of the ovules were poorly developed and aborted, some ovules from a few capsules were well-filled and developed dark brown color, and seem to contain culturable embryos. Embryos, 0.5–3 mm long, were excised from the ovules. These embryos grew vigorously in culture, germinated without callus formation within 4 to 6 weeks and developed shoots and roots simultaneously. Most of the germinated embryos grew into seedlings by 8 to 12 weeks. Through embryo culture, 24 seedlings were obtained from interspecific crosses at Sakai (Table 1) and developed into plants with bulb on MS medium containing 6% sucrose.

In ovule culture of *L. sanguinea* \times *L. incarnata*, no ovules excised from capsules developed to normal size;

Table 1. Results of pollination and *in vitro* culture obtained at Sakai, Osaka.

Cross combinations		(Male) ♂	(Female) ♀		No. of embryos or ovules		No. of seedlings obtained ^y
(♀)	(♂)	Pollen germination rate (%)	No. of florets pollinated	Capsule formation (%) ^z	Cultured <i>in vitro</i>	Germinated	
<i>L. incarnata</i> × <i>L. sprengeri</i>		52.0	35	94.3	4 (embryo)	4	4
× <i>L. pumila</i> ^x		61.8	56	60.7	4 (embryo)	4	3
× <i>L. aurea</i>		50.5	40	80.0	2 (embryo)	1	1
× <i>L. sanguinea</i>		56.6	177	75.7	20 (embryo)	20	16
<i>L. sanguinea</i> × <i>L. incarnata</i>		0.5	16	50.0	80 (ovule)	0	0
<i>L. incarnata</i>	self	2.0	42	88.1	90 (ovule)	0	0
<i>L. sanguinea</i>	self	46.6	10	90.0	27 (ovule)	19	18

^z (No. of capsules developed / No. of florets pollinated) × 100, 20-30 days after pollination.

^y After 8-12 weeks of culture.

^x *L. radiata* var. *pumila* was referred as *L. pumila* in this study.

Table 2. Chromosome number and karyotype of interspecific hybrids and their parental plants.

Chromosome number and karyotype of parental plants		Hybrids		No. of plants observed
(♀)	(♂)	Chromosome number	Karyotype	
<i>L. incarnata</i> 2n=30(4M+3T+22A+1m) ^z	× <i>L. sanguinea</i> 2n=22(22A)	2n=41	4M+3T+33A+1m	16
	× <i>L. sprengeri</i> 2n=22(22A)	2n=41	4M+3T+33A+1m	4
	× <i>L. pumila</i> 2n=22(22A)	2n=41	4M+3T+33A+1m	5
	× <i>L. aurea</i> 2n=14(8M+6T)	2n=37	8M+6T+22A+1m	1

^z M: metacentric chromosome, A: acrocentric chromosome, T: telocentric chromosome, m: small metacentric chromosome.

no hybrid plant was obtained from this cross (Table 1).

In self-pollinations, progenies were only obtained from self-pollination of *L. sanguinea*, although normal capsules formed in *L. incarnata* and *L. sanguinea* (Table 1).

Characterization of hybrid plants

a. Chromosome number and karyotype

Chromosome number and karyotype of parental plants were the standard ones for each corresponding species (Table 2). Chromosome number of *L. incarnata* (2n=30) × *L. sanguinea* (2n=22), *L. incarnata* × *L. sprengeri* (2n=22) and *L. incarnata* × *L. pumila* (2n=22) were 2n=41, and that of *L. incarnata* × *L. aurea* (2n=14) was 2n=37 (Table 2). In those hybrids, the full set of chromosomes (4M+3T+22A+1m) from the female parent (*L. incarnata*) and one set of meiotic chromosomes from each of the male parents were observed, suggesting that 2n + n transmission occurred (Table 2, Fig. 1). Thus, all hybrid plants obtained were triploids.

b. Isozyme analysis

Results of isozyme analysis for APT and GOT revealed that APT isozyme (monomer) bands from both parents were observed in hybrid plants of *L. incarnata* × *L. sanguinea*, *L. incarnata* × *L. pumila* and *L. incarnata* × *L. aurea* (Fig. 2), while the band pattern of *L. incarnata* × *L. sprengeri* was identical to that of *L. incarnata*. For GOT isozyme (dimer) of *L. incarnata* × *L. sprengeri*, one hybrid band as well as bands from both parents, are present (Fig. 2), although band patterns of hybrids from three other combinations were the same as those of *L. incarnata* (female). The above results show that all the 26 plants obtained from interspecific crosses were true hybrids.

c. Characters in hybrid plants of *L. incarnata* × *L. pumila*

The leaves and bulbs of the hybrid plants resembled those of *L. incarnata*, but leaf bud break of the hybrid

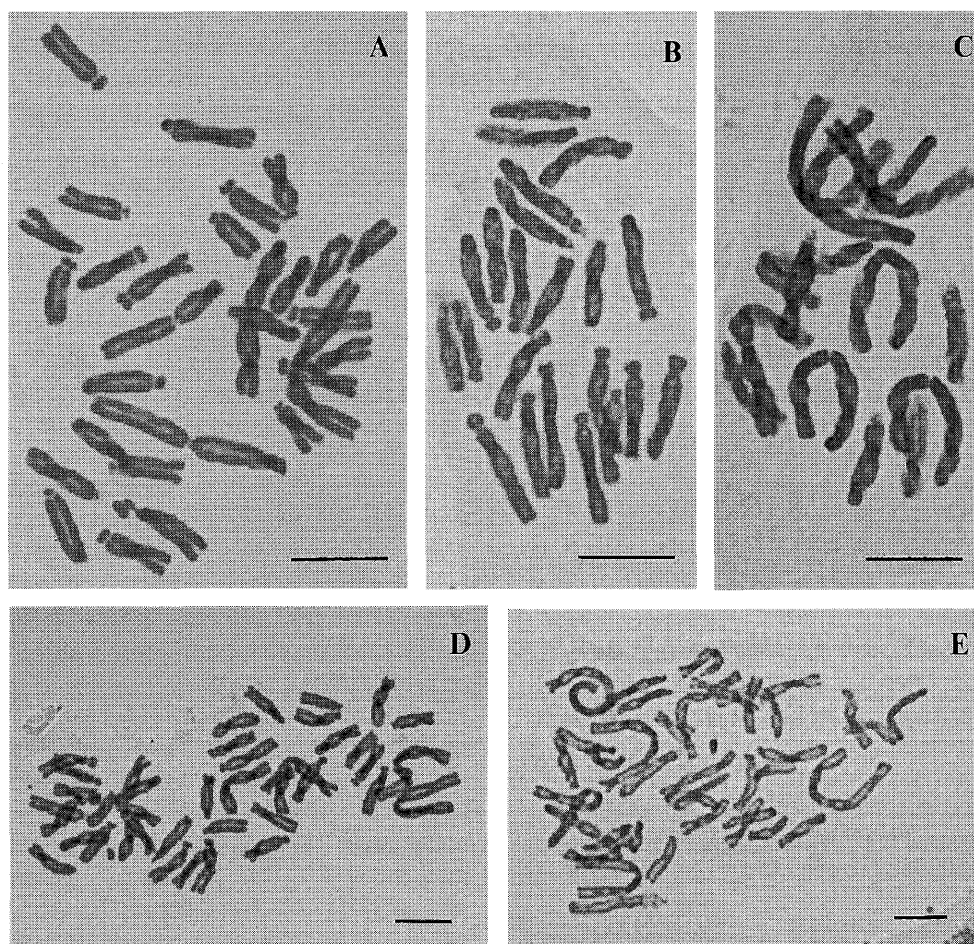


Fig. 1. Metaphase chromosomes in root tip cells. A: *L. incarnata*, $2n=2x=30$ (4M+3T+22A+1m); B: *L. sanguinea*, $2n=2x=22$ (22A); C: *L. aurea*, $2n=2x=14$ (8M+6T); D: *L. incarnata* \times *L. sanguinea*, $2n=3x=41$ (4M+3T+33A+1m); E: *L. incarnata* \times *L. aurea*, $2n=3x=37$ (8M+6T+22A+1m). Bar=10 μ m.

Table 3. Several characters in hybrid plants of *L. incarnata* \times *L. pumila* and their parental plants.

Character	<i>L. incarnata</i>	<i>L. incarnata</i> \times <i>L. pumila</i> ²	<i>L. pumila</i>
Leaf color	gray green	gray green	deep green
Leaf shape	broad linear	broad linear	linear
Shape of leaf apex	obtuse	obtuse	obtuse
Leaf texture	soft	soft	tough
Leaf bud break	spring	autumn	autumn
Bulb shape	ellipsoidal	ellipsoidal	globose
Bulb color	yellow brown	yellow brown	black brown

²Hybrid plants were transplanted to the field and cultivated for seven years.

plants was closer to that of *L. pumila* (Table 3).

Discussion

The first production of an interspecific hybrid using *L. incarnata* was reported by Terada (1970), in which *L. incarnata* was noted to be fertile. However, according to Bose (1958) and Kurita (1987), *L. incarnata* is a sterile species with the heterokaryotype of 4M + 3T + 22A +

1m. In our study, the karyotype of *L. incarnata* (Table 2, Fig. 1) was the same as that of Kurita (1987). No plants were obtained from self-pollination of *L. incarnata*, and no hybrids were obtained by using *L. incarnata* as a male parent (Table 1). These results show that *L. incarnata* plants used in this study are sterile because of low pollen fertility.

Embryo rescue techniques accompanied with tissue

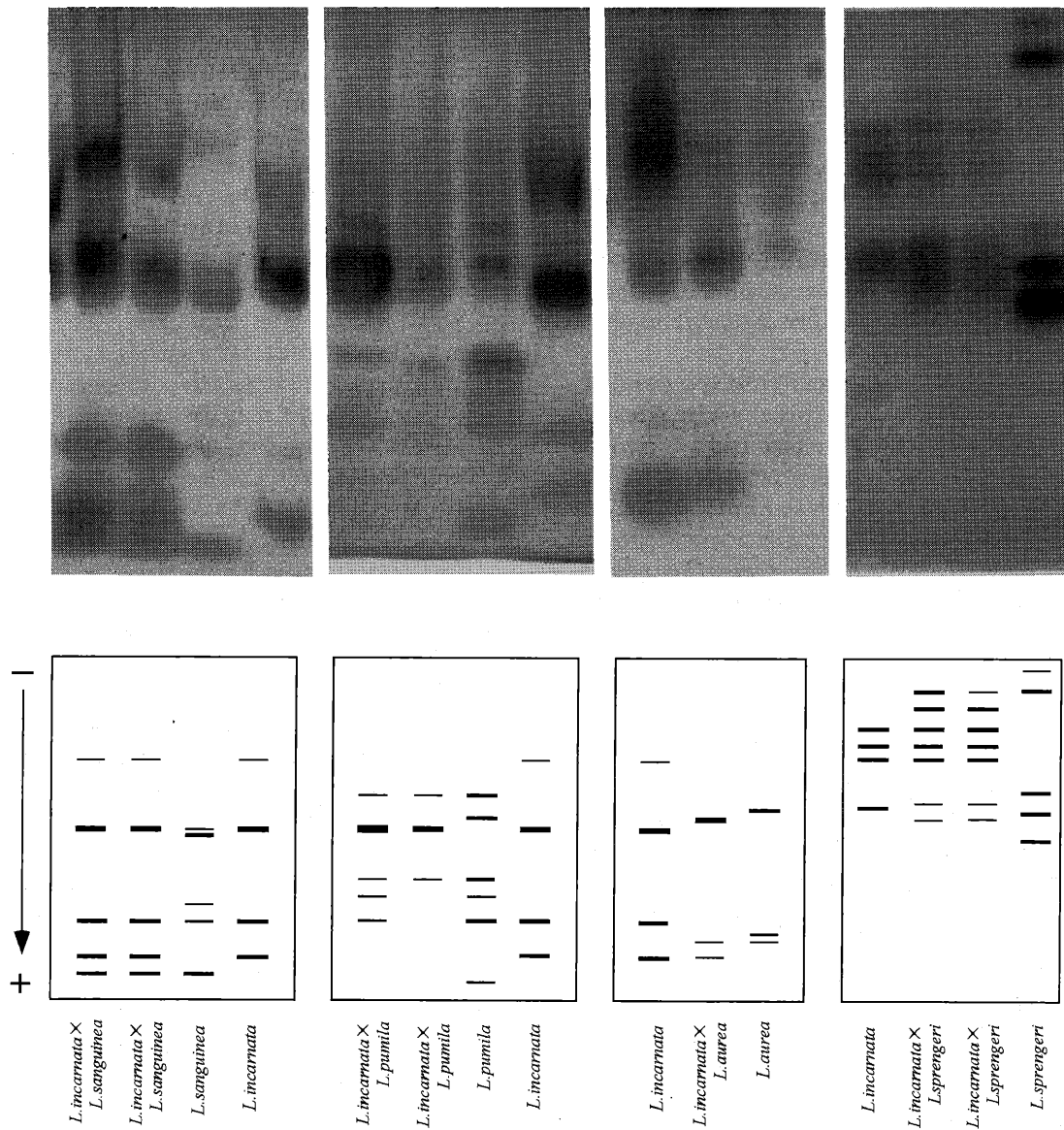


Fig. 2. Zymograms for APT and GOT.

culture are common approaches for producing hybrids between distantly-related species. Few studies on interspecific hybridization in *Lycoris* have been carried out, and ovary culture followed by embryo culture (Isobe and Yazawa, 1993) and ovule culture (Ma et al., 2001) have been developed for obtaining interspecific hybrids in *Lycoris*, especially in the crosses using sterile species that failed to produce hybrid plants without any artificial treatment after pollination. Successful production of seedlings through embryo culture largely depends upon the developmental stage of embryos and the composition of the medium (Sharma et al., 1996). A modified MS medium containing 0.5 mg/l NAA, 5% sucrose, and coconut milk was used in Isobe and Yazawa (1993), but the efficiency for hybrid production was low (0.04 plants per floret in *L. radiata* × *L. sprengeri*). In this study, the modified MS medium with CH, as well as higher levels of potassium and calcium and a lower level of ammonium, was used for embryo and/or ovule culture

because it proved to be useful in ovule culture of interspecific hybrids using several sterile *Lycoris* species (Ma et al., 2001). Hence, the embryos excised from crossed ovules grew well on this medium; 50 to 100% of them developed into seedlings, and many interspecific hybrid plants were finally obtained (Table 1). The results suggest that the modified MS medium of Ma et al. (2001) would be applicable for embryo rescue in interspecific hybridization using sterile *Lycoris* species as female parents.

Formation of triploids from crosses between diploids has also been reported for lily (van Tuyl et al., 1989; Fernández et al., 1996), citrus (Esen and Soost, 1971), taro (Okada and Hambali, 1989) and tomato (Lapidot et al., 1994). The above triploids were assumed to be derived from fusion between an unreduced diploid gamete and a normal haploid one. In this study, karyotype analysis revealed that the triploid hybrids contained the full set of chromosomes of *L. incarnata* and one set

of meiotic chromosomes of male parents. Thus, the triploid hybrids were considered to be derived from fusion of an unreduced $2n$ -egg of *L. incarnata* and a normal haploid gamete of each male parent, similarly as in lily (van Tuyl et al., 1989) etc..

Isozyme analysis has commonly been used to confirm hybridity (Fernandez et al., 1996; Metwally et al., 1996; Honda and Tsutsui, 1997). APT zymograms of *L. incarnata* × *L. sanguinea*, *L. incarnata* × *L. pumila*, and *L. incarnata* × *L. aurea* and GOT zymogram of *L. incarnata* × *L. sprengeri* manifested that these hybrid plants were true interspecific hybrids.

Interspecific hybrid plants from crosses between *L. incarnata* and each of four fertile species were produced through embryo culture using the modified MS medium of Ma et al. (2001). The hybridity of those plants was confirmed by cytological studies and isozyme analysis. This is the first record in producing interspecific hybrid plants using the sterile species, *L. incarnata*. For producing much more interspecific hybrid plants, however, further studies on the more precise time of embryo rescue and medium composition for their culture are needed.

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胚培養を用いた不稔種 *Lycoris incarnata* を交配親とする種間雑種の作出

馬 彪¹・樽本 勲¹・中村典義²・国武久登³

¹大阪府立大学大学院農学生命科学研究科 599-8531 大阪府堺市学園町1-1

²佐賀県農業試験研究センター 840-2205 佐賀郡川副町南里1088

³九州東海大学農学部 869-1404 熊本県阿蘇郡長陽村河陽

摘 要

*Lycoris*属における切花用品種の育種を目的として、不稔種 *L. incarnata* と可稔種 *L. sanguinea*, *L. sprengeri*, *L. radiata* var. *pumila* および *L. aurea* との種間交雑を行った。交雑胚を受粉後 20-30 日目に摘出し、500 mg・liter⁻¹ カゼイン加水分解物を添加した改変 MS 培地 (Ma et al., 2001) で培養した。*L. incarnata* を母本とした 4 組合せにおいて種間雑種を得たが、*L. sanguinea* × *L.*

incarnata では雑種個体を得ることができなかった。細胞学的研究により、種間雑種個体はすべて三倍体であり、それらは母本である *L. incarnata* の非還元配偶子 (2n-egg) と父本の配偶子 (n) との受精から形成されたものと推測された。この染色体観察に加えて、種間雑種個体のアイソザイム分析と特性観察からも、得られた種間雑種個体の雑種性が確認された。