Temperature Effects on Interspecific Hybridization between *Gladiolus* $\times$ *grandiflora* and *G. tristis*

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Abstract. Interspecific hybridization between a modern cultivar of *Gladiolus* $\times$ *grandiflora* hort. ($2n = 60$) and the wild species *G. tristis* L. ($2n = 30$) was made to introduce characteristics of the wild species into the cultivated one. *Gladiolus* $\times$ *grandiflora* is a summer-flowering species, and *G. tristis* flowers in winter. The effect of storage temperature on pollen viability was tested, as long-term storage of pollen was necessary to facilitate crossing these two species. Pollen of *G. tristis* could be stored at -20°C for ≈ 1 year, and was more practical than storage at -80°C . Air temperature affected pollen tube growth, fertility, and fruit set in the cross between *G.* $\times$ *grandiflora* and *G. tristis*, and low temperatures (15 to 20°C) were best. The morphological data and flow cytometric analysis showed that the F_1 plants were hybrids between *G.* $\times$ *grandiflora* and *G. tristis*.

The genus *Gladiolus* is classified in the family Iridaceae, and the species are found in southern Africa, tropical Africa, Madagascar, and Eurasia. The number of species in the genus currently totals 255 (Goldblatt and Manning, 1998). Modern cultivars of *G.* $\times$ *grandiflora*, an important cut flower, were bred originally from only six species (Barnard, 1972), and considerable genetic potential exists for developing new kinds of gladiolus using wild species. As many wild species flower during winter and modern cultivars of *G.* $\times$ *grandiflora* flower in summer, storing the pollen is necessary to facilitate hybridization. In addition, flower abortion of *G.* $\times$ *grandiflora* occurs in the winter because of low light intensity, and the species is not cold-resistant (Imanishi, 1989). Winter-flowering wild species are tolerant of low light intensity and cold temperatures. We attempted interspecific hybridization between *G.* $\times$ *grandiflora* and *G. tristis* in order to introduce some characters of the latter species, such as winter flowering, cold tolerance, and fragrance into the former hybrid. This cross combination has been reported in Israel with some miniature cultivars (Cohen and Barzilay, 1991). However, obtaining the interspecific hybrid

without embryo rescue has been difficult in Japan (Takatsu et al., 1996). Pollen tube elongation and fertilization were observed, but the seed did not develop normally, suggesting that postfertilization barriers prevent interspecific crossing. The effect of environment on these barriers is not clear.

We describe here the long-term storage of pollen and the effect of air temperature on interspecific hybridization of gladiolus, and also provide an estimate of the ploidy level of F_1 progeny by flow cytometry in order to select hybrid plants.

Materials and Methods

Plant materials and hybridization. Seeds of the diploid species *Gladiolus tristis* ($2n = 30$) were purchased from Silverhill Seeds Co. (Capetown, South Africa). This species was grown in a greenhouse at 12 to 25°C during winter and flowered at a low light intensity (≈ 500 to $900\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). The flowers of this species have excellent fragrance. Pollen was collected and predried by the modified procedure of Koopowitz et al. (1984). Pollen collected from freshly dehiscent stamens was dusted on to powder paper ($81\ \text{cm}^2$), predried in a desiccator for 24 h at room temperature, and then stored at -20 or -80°C for 1 year. Pollen viability was evaluated at 1, 3, 6, and 12 months after the initiation of storage, as described by Heslop-Harrison and Heslop-Harrison (1970), using fluorescein diacetate. In addition, the viability of pollen stored for 6 months was confirmed by self-pollination of *G. tristis* in a greenhouse.

Commercial gladiolus cv. Traveller of the tetraploid species *G.* $\times$ *grandiflora* ($2n = 60$)

was potted in April and grown in an open field. Just before the flowering stage in July, potted plants were moved to growth chambers maintained at 15 , 20 , 25 , and 30°C , and were illuminated at $250\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ using a 16-h photoperiod. Three potted plants were used for each treatment. Pollen of *G. tristis* stored for 6 months was used for interspecific crossing. 'Traveller' was self-pollinated under the same conditions as the control. Germination, elongation of pollen tube, and fertilization were confirmed by the methods described by Marubashi and Nakajima (1981) at 12 h, 24 h, 2 d, 3 d, 4 d, and 5 d after pollination, using five florets. Fertility [(number of fertilized ovules/total number of ovules) $\times 100$] was measured each time. The final rate of fruit set [(enlarged pods with matured seeds/total number of pods) $\times 100$] was calculated at 30 d after pollination. Matured seeds were sowed immediately, and were grown as putative hybrid seedlings (F_1 seedlings).

Confirmation of ploidy level of F_1 plants. The ploidy level of the F_1 seedling was determined by chromosome counting and flow cytometry. Three root tips were collected and immersed in 3 mM 8-hydroxyquinoline for 6 h at 20°C , and were fixed in 3 ethanol : 1 acetic acid (by volume) for 24 h at room temperature. They were then hydrolyzed for 8 min in 1 N HCl at 60°C , stained with Schiff's reagent (Wako, Osaka, Japan) for 2 h, and were squashed with a cover slip for chromosome counting. Content of DNA was evaluated by flow cytometry of *G.* $\times$ *grandiflora* 'Traveller', *G. tristis*, and F_1 seedlings, and we estimated the ploidy levels. For flow cytometric analysis, we collected 25-mm² portions of the blade of young leaves from these plants. These portions were chopped in 0.5 mL nuclear extraction buffer (solution A of the High Resolution Kit for Plant DNA; Partec, Münster, Germany). After filtration through a 30- μm nylon sieve, 2 mL of staining solution containing the dye 4,6-diamidino-2-phenylindole-2HCl (DAPI, solution B of the Kit) was added. The analysis was made using a PA flow cytometer (Partec), in three independent trials.

Results and Discussion

Long-term storage of pollen. The viability of pollen of *G. tristis* stored at -20 and -80°C for 6 months was 51.6% and 80.8%, respectively (Fig. 1). This pollen was functional in controlled hybridizations; 36.6 ± 5.8 and 37.6 ± 2.5 seeds per pod were produced using pollen stored at -20 and -80°C , respectively, with no significant difference between treatments. Koopowitz et al. (1984) reported that pollen of *G. tristis* can be stored at -40°C and that stored pollen is viable for 1 year. Our results agree with this study. The viability of pollen stored for 1 year was 27.6% and 67.2% at -20 and -80°C , respectively. Usually, we collect pollen of *G. tristis* in the winter and store it for 6 months before using in hybridizations in the summer (Takatsu et al., unpublished data). These observations suggest that storage of pollen at -20°C is appropriate to maintain its viability for practical use, and is

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more convenient than storage at -40 and -80 °C, as we can use a standard freezer for storage.

Effect of air temperature on success of interspecific crosses. Air temperature had a marked effect on fertility in controlled crosses (Fig. 2). At 20 °C, the pollen tube elongated very rapidly, fertilization was accomplished 12 h postpollination, and the fertility increased to 89.9% 5 d postpollination. At 15 and 25 °C, pollen tube elongation slowed, fertilization was first observed 3 and 1 d postpollination, and the final fertility increased to only 76.4% and 73.2%, respectively. At 30 °C, pollen tube elongation was inhibited more and the final fertility was very low (1.4%). The final rate of fruit set decreased from 73.1% to 0.0% as the temperature increased, but pollen tube growth and fruit set were found even at 30 °C following self-pollination of 'Traveller' (Fig. 3). Crossing barriers frequently occur in interspecific crosses, but sexual barriers preventing crossing have been separated into pre- and postfertilization barriers (Van Tuyl, 1997). In this study, the results of self-pollination of 'Traveller' indicated that female fertility is good at 30 °C. Although fertilization was observed in interspecific crossing at 30 °C, no fruit set was obtained. The best temperature for fertilization was 20 °C (Fig. 2), but that for fruit set was 15 °C (Fig. 3). These results show that postfertilization barriers inhibit interspecific hybridization between 'Traveller' and *G. tristis*, and may explain the failure to obtain hybrids without embryo rescue (Takatsu et al., 1996). Our results suggest that lower air temperatures (15 to 20 °C) increase fertility and may be appropriate to help overcome postfertilization barriers when hybridizing *G. ×grandiflora* and *G. tristis*. Humidity and air temperature is very high (over 30 °C) in summer in Japan; thus, crossing in autumn is preferred if the flowering time can be controlled.

Production of interspecific hybrids. Since the first report by Heller (1973), flow cytometry has been used in plant science (Bergounioux et al., 1992; Galbraith, 1989). It is especially useful for easy, rapid, and accurate determination of ploidy level. Table 1 summarizes the results of chromosome counting and flow cytometric analysis. The chromosome number correlated with fluorescence intensity ($r = 0.98^{**}$) in *G. ×grandiflora* and *G. tristis*, and the diploid species *G. tristis* showed one-half the fluorescence intensity of the tetraploid species *G. ×grandiflora*. This suggests that estimating the ploidy levels of hybrids between these two species by flow cytometry is possible.

Eighty-four seeds matured completely, and 28 seedlings (33.3%) grew after sowing, but only 18 plants (21.4%) flowered normally. F_1 seedlings obtained by interspecific hybridization showed intermediate value of fluorescence intensity between *G. ×grandiflora* and *G. tristis* by flow cytometric analysis (Table 1). Table 2 lists descriptive data for the F_1 plants, whose chromosome number was 45. Most F_1 plants exhibited some degree of male sterility. F_1 plants had intermediate floret num-

Fig. 1. Viability of pollen stored at -20 and -80 °C was measured at 0, 1, 3, 6, and 12 months after storage. Pollen of *G. tristis* was predried at room temperature for 24 h prior to storage. Viability of pollen was evaluated using fluorescein diacetate, as described by Heslop-Harrison and Heslop-Harrison (1970), in three independent trials at each time. Vertical bars indicate SD ($n = 3$).

Fig. 2. Effects of temperature and time on percentage of fertility [(number of fertilized ovules/total number of ovules) $\times$ 100] following pollination of *G. ×grandiflora* flowers with *G. tristis* pollen. Fertilization of ovules was confirmed by the methods described by Marubashi and Nakajima (1981). Vertical bars indicate SD ($n = 5$).

Fig. 3. Final rate of fruit set [(number of enlarged pod with matured seeds/total number of pod) $\times$ 100] at 15, 20, 25, and 30 °C, in interspecific hybridization between *G. ×grandiflora* 'Traveller' and *G. tristis* (Interspecific), and in self-pollination of 'Traveller' (Self). Fruit set was confirmed at 30 d after pollination. Vertical bars indicate SD ($n = 5$).

Table 1. Comparison of flow cytometric analysis, chromosome number determination and estimated ploidy level in a F₁ hybrid between *G. ×grandiflora* and *G. tristis*.

Species	Fluorescence intensity ^z	Chromosome number ^y	Estimated ploidy levels ^x
<i>G. tristis</i>	138.8 ± 3.7 ^w	30	2×
F ₁ seedling	226.7 ± 13.6	45	3×
<i>G. ×grandiflora</i>	286.4 ± 2.1	60	4×

^zFluorescence intensity indicates a relative value of amount of DNA. Within one sample, a minimum of 10,000 particles (total count) were analyzed in three independent trials.

^yChromosome numbers of *G. tristis* and *G. ×grandiflora* have been published. That of F₁ seedling was confirmed by chromosome counting.

^x2×, 3×, and 4× indicate diploid, triploid, and tetraploid, respectively.

^wMean ± SD (*n* = 3).

Table 2. Some characteristics of *G. ×grandiflora* ‘Traveller’, *G. tristis*, and F₁ hybrid.

Strains	Petal Main color	Florets		Leaves		Total height ^y (cm)	Flowering time
		No./stalk ^z	Width (mm)	Total	Width (mm)		
Traveller	Pinkish white	17.0 ± 1.0	10.8 ± 0.3	9.3 ± 0.6	3.2 ± 0.3	138.6 ± 4.9	June to Nov.
<i>G. tristis</i>	Pinkish white	3.6 ± 0.6	3.3 ± 0.6	3.6 ± 0.6	0.6 ± 0.2	90.3 ± 4.5	Feb. to Apr.
F ₁ hybrid	Pinkish white	10.4 ± 2.4	6.2 ± 0.8	6.4 ± 1.1	1.6 ± 0.3	84.8 ± 8.3	Mar. to Oct.
	Vivid pink						
	Dark pink						
	Light pink, etc.						

^zMean ± SD (*n* = 10).

^yFlowering stem length in *G. tristis*.

bers, floret width, leaf number, and leaf width. The total height was measured as the spike length in *G. ×grandiflora* and hybrids, but *G. tristis* has a long flowering stem instead of a spike, indicating that the F₁ plants were hybrids of these parents. These plants showed a variety of flower colors [vivid pink, light pink, and light pink (with or without) a white stripe], but they lacked fragrance.

In this study, we demonstrated that long-term storage of pollen of wild gladiolus at –20 °C is possible, and that low temperature has a positive effect on interspecific crossing. These results suggest that interspecific hybridization between a modern cultivar and a wild species can be made more efficiently with these techniques and can be a useful tool to create new products for the cut flower market.

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