Temperature Effects on Interspecific Hybridization between *Gladiolus xgrandiflora* and *G. tristis*

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Abstract. Interspecific hybridization between a modern cultivar of *Gladiolus xgrandiflora* 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 xgrandiflora* 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. xgrandiflora* and *G. tristis*, and low temperatures (15 to 20 °C) were best. The morphological data and flow cytometric analysis showed that the F₁ plants were hybrids between *G. xgrandiflora* 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. xgrandiflora*, 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. xgrandiflora* flower in summer, storing the pollen is necessary to facilitate hybridization. In addition, flower abortion of *G. xgrandiflora* 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. xgrandiflora* 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 miniatures 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 F₁ 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 μmol·m⁻²·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 dehisced stamens was dusted on to powder paper (81 cm²), 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. xgrandiflora* (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 μmol·m⁻²·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] × 100] was measured each time. The final rate of fruit set [enlarged pods with matured seeds/total number of pods] × 100) was calculated at 30 d after pollination. Matured seeds were sown immediately, and were grown as putative hybrid seedlings (F₁ seedlings).

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...
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**₂ plants had intermediate floret num-

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**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 \([\text{number of fertilized ovules/total number of ovules} \times 100]\) 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 \([\text{number of enlarged pod with matured seeds/ total number of pod} \times 100]\) 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 | Chromosome number | Estimated ploidy levels |
|-----------------|------------------------|-------------------|-------------------------|
| G. tristis      | 138.8 ± 3.7*           | 30                | 2x                      |
| F1 seedling     | 226.7 ± 13.6           | 45                | 3x                      |
| G. ×grandiflora | 286.4 ± 2.1            | 60                | 4x                      |

*Fluorescence 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.

Table 2. Some characteristics of G. ×grandiflora 'Traveller', G. tristis, and F1 hybrid.

| Strains         | Petal color     | No./ Width | Total Width | Total Height | Flowering time |
|-----------------|-----------------|------------|-------------|--------------|----------------|
| Traveller       | Pinkish white   | 17.0 ± 1.0 | 9.3 ± 0.6   | 138.6 ± 4.9  | June to Nov.   |
| G. tristis      | Pinkish white   | 3.6 ± 0.6  | 3.6 ± 0.6   | 90.3 ± 4.5   | Feb. to Apr.   |
| F1 hybrid       | Pinkish white   | 10.4 ± 2.4 | 6.4 ± 1.1   | 84.8 ± 8.3   | Mar. to Oct.   |

| Strains         | Petal color     | No./ Width | Total Width | Total Height | Flowering time |
|-----------------|-----------------|------------|-------------|--------------|----------------|
| Traveller       | Vivid pink      | 17.0 ± 1.0 | 9.3 ± 0.6   | 138.6 ± 4.9  | June to Nov.   |
| G. tristis      | Light pink      | 3.6 ± 0.6  | 3.6 ± 0.6   | 90.3 ± 4.5   | Feb. to Apr.   |
| F1 hybrid       | Dark pink       | 10.4 ± 2.4 | 6.4 ± 1.1   | 84.8 ± 8.3   | Mar. to Oct.   |

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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