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Interspecific hybridization between *Tigridia pavonia* and *T. augusta* through ovary slice culture

J.-L. Piña-Escutia[1], L.-M. Vázquez-García[2] and A.-M. Arzate-Fernández[1]

[1]Laboratorio de Biología Molecular Vegetal,
Centro de Investigación y Estudios Avanzados en Fitomejoramiento,
Facultad de Ciencias Agrícolas, Universidad Autónoma del Estado de México,
Toluca, Estado de México, México
[2]Centro Universitario Tenancingo, Ex-Hacienda de Santa Ana,
Universidad Autónoma del Estado de México, Tenancingo,
Estado de México, México

Corresponding author: A.-M. Arzate-Fernández
E-mail: amaury1963@yahoo.com.mx

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ABSTRACT. *Tigridia pavonia* is the most popular species in the *Tigridia* genus, and is currently marketed in Europe, Asia, and Australia as a landscape plant. Although it is native to Mexico, there are no breeding programs for it. In this study, we attempted to increase its flower color spectrum and growth habit by interspecific hybridization with *T. augusta*. Interspecific hybrids between *T. pavonia* and *T. augusta* were successfully obtained for the first time using the cut-style pollination and ovary slice culture techniques. On the contrary, no hybrids were obtained from a reciprocal cross. At three, four, and five days after pollination (DAP) ovaries were sliced and cultured on Murashige and Skoog medium without growth regulators and ammonium nitrate, but were supplemented with 6% sucrose, 50 mg/L yeast extract, and 0.25% Gelrite. After 80 days of culture initiation, the germination of only 10 embryos was observed in ovary slices cultured at three DAP. After

transfer to identical fresh medium, six hybrid embryos developed into seedlings. All obtained hybrid seedlings were transplanted successfully to soil, and grew normally. The progenies investigated were identified as true hybrids based on randomly amplified polymorphic DNA analysis.

Key words: *Tigridia pavonia*; *T. augusta*; Cut-style pollination; Ovary slice culture; Interspecific hybrids; Genetic analysis

INTRODUCTION

Interspecific hybridization has been used as a potential tool for inducing variability and the introgression of desirable new traits such as novel flower color among several crops. However, in distant hybridization, the barriers may be prezygotic, postzygotic, or both, and are likely to be more severe in crosses between more distantly related taxa than in those between closely related taxa (Ladizinsky, 1992).

Typically, embryo rescue and ovule culture have been effectively used to overcome genetic incompatibility and develop interspecific hybrids in several crops (Morgan, 2004; Kaushal et al., 2005; Eeckhaut et al., 2007; Vlachostergios et al., 2007; Hoshino et al., 2008). Nevertheless, both techniques are not very useful when pro-embryos are used, as they can be damaged during excision due to their very small size. Thus, ovary-slice culture (OSC) has been a good option for successfully culturing embryos in an earlier phase (Arzate-Fernández et al., 1998; Umehara et al., 2006; Wang et al., 2009).

The genus *Tigridia* consists of at least 45 species, of which 37 are native to Mexico. These species comprises plants with curiously formed and highly colored flowers that exhibit great morphological variation in color, shape, and structure, making many species potentially valuable as cultivated plants (Rodríguez and Sytsma, 2006).

Tigridia pavonia (L.f.) DC. is native to Mexico. This plant grows up to a height of 30-150 cm, with numerous basal leaves 20-50 cm in length. Spathes are 6 cm in length. Flowers may be pink, orange, red, yellow, or white, with spots or blotches towards the center. Tepals extended 7-10 cm x 4-6 cm, creating a broad calyx (Vázquez-García et al., 2001). Due to the great variability in color and the beauty of its flower, *T. pavonia* is the most popular species in the *Tigridia* genus, and this species is currently widespread in Europe, Asia, and Australia, where is commercialized as a landscape plant.

On the other hand, *T. augusta* Drapiez is also endemic to Mexico. This plant grows up to a height of 5-30 cm, with lanceolated leaves 8-40 cm in length. Spathes are 3 cm in length. Flowers are 3-5 cm in diameter, with an attractive purple color with yellow spots towards a white center. So, these features make it potentially ornamental.

Molseed (1970) carried out a project to apply distant hybridization in a conventional way to improve *Tigridia*. However, no satisfactory results were obtained due to semi-compatibility among *Tigridia* species. Although hybridization between *T. pavonia* and *T. augusta* (both with 2n = 28) could potentially combine the ornamental characteristics of *T. pavonia*, such as plant height and wide flower color spectrum with the flower color and growth habit of *T. augusta*, hybridization between these two species has not yet been reported.

The aim of the present study was the production of interspecific hybrids between *T. pavonia* and *T. augusta* by using OSC. In addition, the hybridity of the seedlings obtained was tested using RAPD analysis.

MATERIAL AND METHODS

Plant material

Reciprocal crosses between *T. pavonia* and *T. augusta* were carried out in a greenhouse. Flowering of both parents (Figure 1a and b) was synchronized by controlling irrigation. The flowers of both species were emasculated in the morning before anthesis, and the stigmas were pollinated manually with the respective pollen. Two pollination methods were tested: cut-style pollination (CSP) and without cut-style pollination (WCSP) as described by Arzate-Fernández et al. (1998). After pollination, the flowers were labeled and covered with butter paper bags.

Figure 1. Parents used in the present study. **a.** *Tigridia pavonia* L.f. (DC) var. Carolina. **b.** *T. augusta* Drapiez.

In vitro culture procedure

Five ovaries from each cross were harvested at 3, 4, and 5 days after pollination (DAP), and disinfected as proposed by Piña-Escutia et al. (2010). After disinfection, the OSC procedure was conducted according to Arzate-Fernández et al. (1998). Briefly, the top and bottom parts of each ovary were discarded, and only the middle parts of the ovaries were sliced. Two to five disks (2-4 mm thick) were obtained from each ovary. Each disk was considered as an explant, and each explant contained 20 ovules on average.

After sectioning, five explants were placed horizontally on Petri dishes (90 x 90 mm) containing 30 mL culture medium. For embryo germination, a modified hormone-free Murashige and Skoog (MS) medium was used as described by Arzate-Fernández et al. (1998). The pH of the medium was adjusted to 6.3 with 1 N NaOH prior to autoclaving at 120°C at 1.1 kg/cm^2, for 20 min.

Ovary slice cultures were incubated in growth chambers at 25° ± 1°C in the dark for three weeks. Later, all the cultures were incubated under cool white fluorescent tubes with a light intensity of 34 ± 5 µmoL m^{-2} s^{-1} and a 16-h photoperiod.

After 80-85 days of culture, germinated embryos were transferred to test tubes (25 x 120 mm) containing 15 mL fresh medium culture. The incubation conditions and photoperiod were the same as before.

The number of regenerated plantlets was measured 100 days after culture initiation.

When the regenerated plantlets were 6-8 cm in height, they were rinsed free of tissue medium culture with distilled water and potted in plastic pots containing a sterilized mixture (1:1) of compost and agrolite. Three weeks later, the plants were transferred to a greenhouse.

RAPD analysis

Two developed plants and their respective parents underwent RAPD analysis to confirm their hybridization. DNA was extracted from 100 mg fresh leaves using the CTAB method. The DNA samples were stored at -20°C prior to RAPD analysis.

All DNA amplifications were performed in a total reaction volume of 10 µL containing 10 ng DNA template, 1X PCR buffer, 1.25 mM $MgCl_2$, 250 µM dNTPs, 0.2 µM primer and 0.1 U Taq DNA polymerase (Mercury, USA). For RAPD reactions, five primers (P628, P635, P647, P496, and P497) were used (Debener and Mattiesch, 1998; Yamagishi et al., 2002).

Amplifications were performed in a thermocycler (Mastercycler gradient; Eppendorf, Germany). The PCR program was carried out according to Debener and Mattiesch (1998) and Yamagishi et al. (2002). The PCR products were subjected to horizontal electrophoresis on 1% agarose gel in 1X TAE buffer at 100 V and 120 mA for 80 min. A 100-3000-bp DNA ladder (MBI Fermentas, USA) was used as a molecular weight marker, and the ethidium bromide-stained gels were visualized using a transilluminator (UVP, USA). The molecular markers generated by the RAPD primers were analyzed in terms of presence or absence of informative bands to confirm the putative hybrids.

RESULTS AND DISCUSSION

In this study, interspecific hybrid production was obtained only in the *T. pavonia* x *T. augusta* cross, whereas no hybrid plant was obtained when *T. augusta* was used as the female parent. For *T. pavonia* x *T. augusta* crosses, a total of 3000 ovules were enlarged and swollen at 20 days after culture initiation (Figure 2a). However, after 100 days of culture, only 10 embryos from 3 DAP and CSP were obtained (Table 1). Thus, while the former yielded normal germination (Figure 2b), all the ovules from 4 and 5 DAP became dark brown and failed to germinate (Figure 2c). Likewise, four of 10 embryos exhibited abnormal development and stopped growing after 100 days of culture (Table 1; Figure 2d), whereas the remainder of the germinated embryos exhibited normal development (Figure 2e). When the regenerated plantlets were 6-8 cm in height, they were potted in plastic pots (Figure 2f) and transferred to greenhouse conditions three weeks later.

It is known that pre-fertilization barriers such as style incompatibility, as well as post-fertilization barriers such as endosperm degeneration are factors that limit the development of interspecific hybrids. Nevertheless, methods such as CSP and ovary slicing have been successfully used to overcome these barriers (Arzate-Fernández et al., 1998; Umehara et al., 2006; Wang et al., 2009). Molseed (1970) carried out interspecific crosses between *T. pavonia* and *T. augusta*; however, seeds could not be obtained, mainly due to a lack endosperm. In the present study, we successfully obtained embryos for the first time using the CSP method and OSC, and developed seedlings from ovary slices at 3 DAP but not at 4 or 5 DAP. Our results are in agreement with those reported by Carrillo-Ocampo and Engleman (2002), who mentioned that although the appearance of the pollen tube in *T. pavonia* can occur from the first day after anthesis (daa), fertilization occurs at 3 daa.

Table 1. Effect of the pollination method and days after pollination on the production of interspecific hybrids between *Tigridia pavonia* and *T. augusta* by ovary-slice culture.

Cross	Pollination method	Days after pollination	Number of			
			Explants inoculated[a]	Embryos germinated	Embryos stopping growing	Seedlings obtained
T. pavonia x *T. augusta*	WCSP	3	25	0	0	0
		4	25	0	0	0
		5	25	0	0	0
	CSP	3	25	10	4	6
		4	25	0	0	0
		5	25	0	0	0
Total				10	4	6
T. augusta x *T. pavonia*	WCSP	3	25	0	0	0
		4	25	0	0	0
		5	25	0	0	0
	CSP	3	25	0	0	0
		4	25	0	0	0
		5	25	0	0	0
Total				0	0	0

WCSP = without cut-style pollination; CSP = cut-style pollination. [a]An ovary slice disc was considered as an explant.

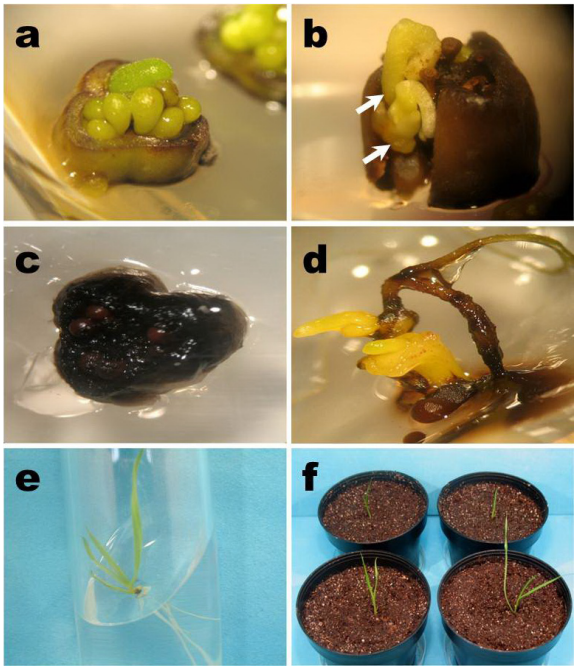

Figure 2. a. *In vitro* development of hybrid ovules from the cross between *Tigridia pavonia* and *T. augusta* [3 days after pollination (DAP)] after 20 days of culture. Note the swollen ovules inside the ovary slice. **b.** Germinated hybrid embryos (arrows) observed at 80 days after culture initiation. **c.** Failure of hybrid embryo germination from a cultured ovary at 5 DAP from the cross between *T. pavonia* and *T. augusta* observed at 80 days of culture. **d.** A germinated hybrid embryo from the cross between *T. pavonia* and *T. augusta* (3 DAP) showing abnormal development and stopped growing after 100 days of culture. **e.** Normal development of a young plantlet of the same cross, showing a well-developed root system, 100 days after culture initiation. **f.** Hybrid plants from the cross between *T. pavonia* and *T. augusta* completely adapted to soil, 120 days after culture initiation.

On the other hand, the failure in hybrid generation with the WCSP method suggests a possible pre-fertilization barrier, considering that the pollen tube of *T. augusta* could not penetrate the long distance of the *T. pavonia* style, and failed to reach the *T. pavonia* ovules. Therefore, this barrier could be overcome successfully only with the use of CSP method, as was observed in this study. Further research, however, would be necessary to confirm this.

Higher osmotic pressure in the medium can enhance the development of younger embryos *in vitro*. Nevertheless, it appears that this depends greatly on the species, because while Liu et al. (2006) showed that 2% sucrose was more efficient for initiating hybrid embryos in *Leucadendron*, Ikeda et al. (2003) reported that *in vitro* embryo development in *Lilium* ovules was influenced significantly by sucrose concentration of 9%. In the present study, the hybrid embryos were obtained in MS medium supplemented with 6% sucrose. Our results are similar to those reported by Arzate-Fernández et al. (1998), who obtained *Lilium concolor* x *L. longiflorum* hybrids by culturing ovary slices on the same medium. Thus, this suggests that a higher osmotic pressure of the medium can be suitable for culturing very young embryos in *Tigridia*, although further attempts will be required to determine the best sucrose concentration and to increase the embryo germination rate.

A fundamental aspect in a cross breeding program is the identification of the true hybrid. The confirmation of hybrid plants in the initial development stage is important for reducing time and costs in the maintenance of the plants (Conceição et al., 2011). Molecular markers have been reliable in genotype characterization because they offer fast screening and more precise discriminatory power (Vicente and Fulton, 2003). Due to short-time requirements and no information on the involved DNA sequences, RAPD markers have been used successfully in the genetic analysis of hybrids in several species such as *Lilium* spp (Yamagishi et al., 2002), *Vanilla* spp (Divakaran et al., 2006), and *Oryza* spp (Hashemi et al., 2009). In the present study, RAPD markers were used to identify the hybridization of the progenies. When the primer P496 was used (Figure 3a), an 850-bp band common to *T. pavonia* and the hybrids was obtained, whereas this band was not detected in *T. augusta*. When the primer P647 was used (Figure 3b), a 1.7-kbp band was detected in both *T. augusta* and the hybrids, but not in *T. pavonia*. Likewise, when the primer P628 was used (Figure 3c), we observed a 1-kbp band from *T. pavonia*, a 1.7-kbp band from *T. augusta*, and a 1.2-kbp band common to both parents. The DNA of regenerated plants contained the three 1-, 1.2- and 1.7-kbp fragments. Thus, the plants obtained possessed both male and female parent-specific bands, and they can be considered interspecific hybrids of *T. pavonia* and *T. augusta*.

In relation to florescence and flower features, the species utilized in the crossings exhibit important characteristics for ornamental uses. *T. pavonia* flowers have 7-10 cm in diameter, which may be pink, orange, red, yellow, or white (Vázquez-García et al., 2001). In addition, they produce flowers for up to 8 weeks and are therefore excellent outdoor plants. On the other hand, *T. augusta* is smaller than *T. pavonia*, which could be important for the reduction of such features and selection of indoor plants. Moreover, *T. augusta* is precocious when compared to *T. pavonia*, because the former is already flowering when *T. pavonia* is still in the vegetative phase.

To date, the growth and leaf morphogenesis of the hybrids obtained in this study are intermediates of the two parental species, but flowering has not been observed. In *Tigridia*, when a plant is regenerated from a seed, flowering is obtained in the second year of growth. Therefore, although characterization of the hybrids is now in progress, the total changes in plant architecture and flower color of the hybrids obtained can only be observed in the second year of growth.

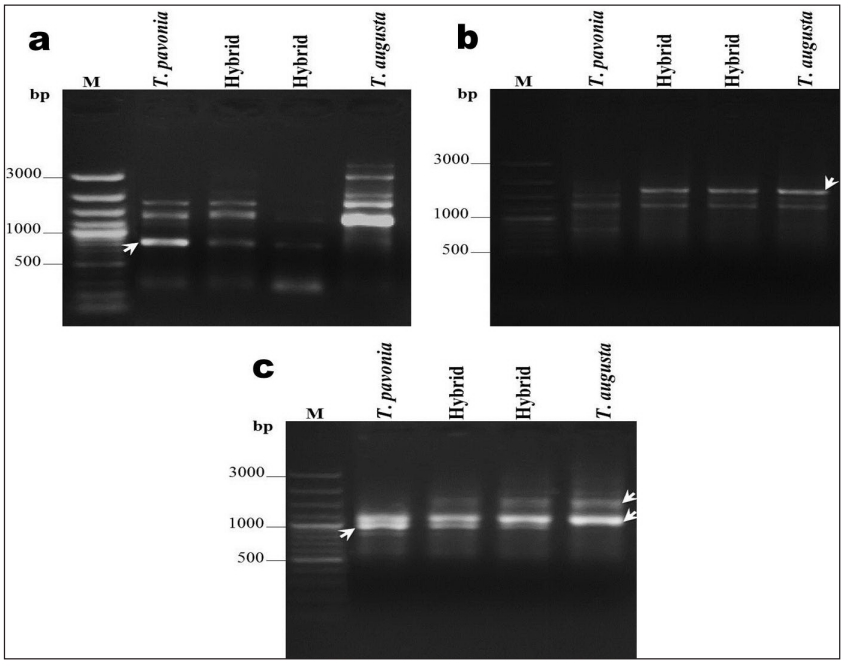

Figure 3. RAPD analysis showing amplification products generated from DNA of *Tigridia pavonia* and *T. augusta*, and their putative hybrids with the primers P496 (**a**), P647 (**b**) and P628 (**c**). Arrows indicate the fragments that were transmitted from each parent to the putative hybrids. *Lane M* = DNA ladder.

CONCLUSIONS

The present study showed that interspecific hybrids production of *T. pavonia* x *T. augusta* by using CSP and the OSC technique was possible. The plants obtained were identified as true hybrids through RAPD analysis. Thus, this study constitutes the first successful report on interspecific hybridization in *Tigridia*, and demonstrates that even when the hybrid number obtained was low, the method used here could be applied to generate new interspecific hybrids among other *Tigridia* species that, by conventional methods, are sexually incompatible and difficult to hybridize.

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