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Effect of Manipulating Flowers In Situ on Sexual Reproduction of *Cyrtopodium macrobulbon* (La llave & Lex.) Romero-González (Orchidaceae) in Tamaulipas, México

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Abstract

Species of the Orchidaceae family, as floristic members of the ecosystems they inhabit, are of great importance. However, many of them have been categorized as at risk because of the low rate of seed production. For this reason, knowledge of the biology of pollination of species at risk is vital for their conservation. Studies conducted in this area are scarce; thus, the objective of this research was to determine the mechanism of pollination for reproductive success in a wild population of *Cyrtopodium macrobulbon* La Llave & Lex. The study was conducted in the ejido Gallos Grandes, Tula, Tamaulipas, Mexico. We selected 23 *Cyrtopodium macrobulbon* individuals in the flowering stage and described the morphology of the flowers and inflorescences, as well as the adult individuals. To determine their mechanisms of pollination, five treatments were set up. The fruits produced by everyone were collected and we obtained their fructification percentage and seed mass. The data were analyzed using SAS 9.0. The percentage of fructification was higher in the control treatment, while the seed mass was significantly higher in the treatment of cross pollination. This study determined effective pollination mechanisms that guarantee reproductive success and that can be considered in conservation plans for the species *C. macrobulbon*.

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1. Introduction

Mexico is home to a remarkable wealth of orchids, which belong to one of the five most diverse vascular plant families; around 1,260 species belonging to 170 genera have been described (Hágsater *et al*., 2005; Soto *et al*., 2007). It is one of the most vulnerable families due to destruction of their habitat and to clandestine extraction, the reasons that 181 species are found in some risk category (SEMARNAT, 2010).

The genus *Cyrtopodium* belongs to the subfamily Epidendroideae, tribe Cymbidieae Pfitzer and subtribe Cyrtopodiinae Benth. (Dressler, 1993; Szlachetko, 1995; Chase *et al*., 2003; Pridgeon *et al*., 2009) and comprises approximately 50 species. The greatest diversity is found in the west central region of Brazil, and the rest is distributed from Florida and Mexico to northern Argentina (Barros *et al*., 2003). The species of the genus are extracted because of their ornamental and ethnopharmacological value (Vieira *et al*., 2000; Neto & Morais, 2003; Barreto & Parente 2006; Brasileiro *et al*., 2006; Romero-González *et al*., 2008). Some species such as *C. andersonii* R. Br. and *C. cardiochilum* Lindl. are used to treat colds, tuberculosis, and hemoptysis (Corrêa, 1975), and in Brazil, to treat inflammatory and immunoregulator disorders (Barreiro *et al*., 2004; Barreto & Parente, 2006). Extracts of the *C. paranaense* Schltr. pseudobulbs are used to cure lesions in general (Pereira & Felcman, 1998; Boscolo & Valle, 2008; Boscolo *et al*., 2010).

C. macrobulbon La Llave & Lex. is widely distributed in Mexico. It grows in tropical climates in rocky substrates where it is continuously exposed to pillage and destruction of its natural habitat (Carranza-Álvarez *et al*., 2015). The pseudobulbs are used as medicine to treat abscesses and as a balm agent in the states of Morelos and Yucatán (Martínez, 1979; Arellano *et al*., 2003; Monroy-Ortiz *et al*., 2013; Garay *et al*., 2018).

Moreover, it has been documented that in Mexico City it is used to treat male urinary infections, and the gum extracted from the pseudobulbs is used as glue in book binding and diverse handcrafts (García-Peña & Peña, 1981).

Floral evolution has been associated with differences in pollination syndromes. In orchids, this is extremely diverse and includes reward and deceit systems (Phillips *et al*., 2017). Species of the genus *Cyrtopodium* do not offer rewards to their pollinators, but they attract bees of the Centridini and Euglossini families by deceit (Chase *et al*., 1992; Van Der Cingel, 2001; Pansarin *et al*., 2008). Regarding orchid reproductive and evolutionary biology, fruit set and seed production are the most important processes used to determine plant reproductive success. For this reason, the importance of pollinators for this family is reflected in their complex pollination mechanisms. In the reproductive system of orchids self-compatibility prevails (Proctor *et al*., 1996; Tremblay *et al*., 2005), and thus, conservation depends on the existence of biotic interactions within the community, for example with mycorrhizal fungi and pollinating organisms. Therefore, the disappearance of one of them would also cause a reduction or loss of natural orchid populations affecting humans to some degree, as in the case of the species of the genus *Cyrtopodium*. The objective of this study is to determine the mechanism of pollination for reproductive success in a wild population of *Cyrtopodium macrobulbon*.

2. Materials and Methods

2.1. Study Area

The study was conducted in the ejido Gallos Grandes, Tula, Tamaulipas, Mexico. The study site has elevations from 1,035 to 1,100 m. The climate is predominantly warm steppe; mean annual temperature is 18 °C, with highs of 43 °C and lows of 1 °C. Soil is regosol from sedimentary rock, and vegetation is rosetophyllous scrub (INEGI, 1999; Treviño-Carreón and Valiente-Banuet, 2005).

From March to September 2018, we visited the study site and selected a total of 23 *Cyrtopodium macrobulbon* La Llave & Lex. reproductive individuals with developed inflorescence.

2.2. Measurement of Cyrtopodium macrobulbon Floral Structures

We measured the *C. macrobulbon* floral structures and described the morphology of the species. To this end, we collected 10 flowers from 10 individuals and took measurements with a Surtek Vernier.

2.3. Pollination Treatments

We established five pollination treatments during the flowering period: a) control, in which the flowers were not manipulated; b) self-cross, in which the pollinia from one flower was placed in another flower of the same individual and later isolated in organza-type bags; c) automatic pollination, flowers were isolated in organza-type bags without removing the pollinias; d) cross pollination, the pollinias were removed, placed in flowers of a different individual, and isolated in organza-type bags; and e) emasculation, in which the pollinias were removed from the flowers. Each pollination treatment was marked with thin wires of different colors.

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2.4. *Analysis of Fructification Data by Pollination Treatment*

The percentage of fruits produced by each treatment was determined. An analysis of variance and a Tukey comparison of means test at 0.05 were performed for the five pollination treatments. The data were analyzed using SAS 9.0.

2.5. *Analysis of Seed Weight Data of the Fruits by Pollination Treatment*

The seeds of the *C. macrobulbon* fruits produced by each treatment were weighed in a Sartorius analytical balance. An analysis of variance and a Tukey comparison of means test at 0.05 were performed and the data were analyzed using SAS 9.0.

3. Results

3.1. *Morphological Description of Cyrtopodium macrobulbon*

Cyrtopodium macrobulbon La Llave & Lex. belongs to the Orchidaceae family. It has a terrestrial growth habit and grows between bare rocks located in open spaces of the low deciduous forest; it is associated with small patches of rosetophyllous scrub affected by the water stress conditions of these environments. Reproductive individuals reach heights of 0.6 to 1.3 m and are found in groups of erect pseudobulbs. The leaves are linear with a pointed apex. The inflorescence is lateral paniculate type and can have more than 100 flowers. The labellum measures 3.0 cm on average, while the column reaches a length of 5.0 mm. The pollinia is 2 mm long on average, while the fruits are capsular with an oblong to ovate shape and can reach up to 10 cm in length.

3.2. *Percentage of Fruits Formed, by Treatment*

According to the results of the pollination treatments (Table 1), the self-cross treatment produced the highest percentage of fruits, followed by the emasculation treatment, which produced 10% less.

Table 1. Percentage of fruit set of the five pollination treatments in *Cyrtopodium macrobulbon*.

Treatments	Flowering plants	Flowers by treatment	Fruit set	Percentage of fruit
Control	28	115	71	52.59
Self-cross	28	97	55	56.70
Self pollination	28	104	57	54.80
Cross pollination	28	115	59	51.30
Emasculation	28	115	49	42.60

The ANOVA for fruit production by treatment (Table 2) indicated that there are statistically significant differences among treatments ($P < 0.05$). Given that the value of P of the treatment test is less than 0.05, there is a statistically significant difference between the fruits of one treatment and those of the next, with a confidence level of 95%. Moreover, this statistically significant difference is also present in the source of flower variation.

Table 2. ANVA results of fruit set with two variation sources: treatments and flowers.

Variation sourse	DF	Mean squares	*F Value*	*P Value*
Treatments	4	2.32	3.53	0.0090
Flowers	1	120.30	182.14	<.0001

P Value < 0.05 = Statistical significance.

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In the test of comparison of means of the five pollination treatments with grouped standard errors (Table 3), the control treatment shows a statistically significant difference with respect to the emasculation treatment. However, the treatments cross pollination, automatic pollination, and self-cross pollination are statistically similar.

Given that the value of *P* of the F test is lower than 0.05 (Table 4), there is a statistically significant difference in percentage between treatments, with a confidence level of 95%.

Table 3. Comparison of the means in the five pollinationtreatments.

Mean	Treatments	Groups	
2.53	Control	A	
2.10	Cross-pollination	A	B
2.03	Self-pollination	A	B
1.96	Self-cross	A	B
1.75	Emasculation		B

Different letters = Statistical significance.

Table 4. ANVA test for percentage of fruits by treatments.

Variation Source	DF	Mean squares	*F Value*	*P Value*
Between groups	4	2148.9	7.90	<.0001
Within groups	287	2719.62		

P Value < 0.05 = Statistical significance.

The comparison of means for the seed mass of the fruits by treatment shows that the treatments control, self-cross and emasculation are significantly different from the cross pollination and automatic pollination treatments (Table 5).

Table 5. Means comparations of the fruits seminal mass of the five pollination treatments.

Mean	Treatments	Groups	
85.142	Control	A	
106.81	Cross-pollination		B
117.82	Self-pollination		B
76.68	Self-cross	A	
72.46	Emasculation	A	

Different letters = Statistical significance.

3.3. Analysis of Fruit Seed Mass, by Treatment

The analysis of variance for seed mass of the fruits from five pollination treatments (Table 6) indicated that there is a statistically significant difference between treatments. The results indicate that there is a statistically significant difference in *C. macrobulbon* seed mass, by treatment, given the value of $P < 0.05$, while the comparison of means for the seed masses from fruits of the five treatments (Table 7) showed that there are differences among treatments.

Table 6. ANVA test for fruits seminal mass by treatment.

Variation Source	DF	Mean squares	*F Value*	*P Value*
Model	4	0.55	3.09	0.0162
Error	287	0.17		

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P Value < 0.05 = Statistical significance.

Table 7. Means comparison of fruits seminal mass of five pollination treatments.

Mean	Treatments	Groups	
4.21	Cross-pollination	A	
4.04	Control	A	B
4.01	Emasculation	A	B
3.99	Self-cross		B
3.97	Self-pollination		B

Different letters= Statistical significance.

The test of comparison of means of the seed mass *C. macrobulbon* of fruits revealed statistically significant differences among the treatment's cross pollination, self-cross pollination, and automatic pollination.

4. Discussion

Fruit production in orchids can be limited by a variety of conditions, such as absence of pollinators, herbivores, or limited resources in the mother plant (Ackerman & Montalvo, 1990).

The results indicate that *Cyrtopodium macrobulbon* is an orchid species that does not reward floral visitors, it is self-compatible but is dependent on pollinators due to a low percentage of self-compatibility. It is necessary to have a pollen vector to transport pollen, even for self-pollination, and cross pollination is favored in most of the species (Van der Pijl & Dodson, 1966; Pansarin, 2003; Mickeliunas *et al*., 2006).

Speciation does not guarantee an adequate rate of fructification in natural conditions since studies on diverse species have shown reproductive success below 10% (Carvalho & Machado, 2002; Martini *et al*., 2003; Pansarin, 2003; Pansarin *et al*., 2006; Mickeliunas *et al*., 2006; Carvalho & Machado, 2006; Pansarin & Amaral, 2008; Pansarin *et al*., 2008a; Pansarin & Pansarin, 2010; Rech *et al*., 2010; Storti *et al*., 2011).

Ackerman (1989) mentions that many fruits may form but can be lost before maturity for several reasons, such as the effect of non-viable pollen, attack by pathogens, mechanisms of post-zygotic incompatibility, and herbivores.

Another reason for low reproductive success in many orchid species is the low frequency of pollinator visits, especially when the species does not offer any type of reward, but has the strategy of floral mimesis, as is the case of *Cyrtopodium polyphyllum* (Vell.) Pabst ex F. Barros., which is very infrequently visited by different bees that are not efficient pollen transferers (Van der Pijl & Dodson, 1966; Pansarin *et al*., 2008). This species has adapted, and some type of abiotic pollination occurs (Pansarin *et al*., 2008b).

Diverse studies have observed that orchids that produce nectar tend to obtain a higher percentage of pollination and production of fruits than those that do not (Ackerman *et al*., 1994; Neiland & Wilcock, 1998; Smithson & Gigord, 2001).

5. Conclusion

Of the pollination treatments, self-cross pollination produced the highest percentage of fruits (56.70%), followed by the emasculation treatment, which had 10% less. Moreover, the analysis of variance indicated that the difference is statistically significant, while the comparison of means showed that the control treatment is statistically different relative to the emasculation treatment. The analysis of variance for seed weight of the five pollination treatments showed that there are statistically significant differences, and the comparison of means revealed that the largest quantity of seed mass was produced by cross pollination, which is therefore an effective method for reproductive

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success of *Cyrtopodium macrobulbon*. The self-cross and automatic pollination treatments show that the species is self-compatible at low rates.

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