Mating system and female reproductive success of the endemic and endangered epiphyte *Rhynchostele cervantesii* (Orchidaceae) in a cloud forest in Michoacan, Mexico

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Background and aims – The Orchidaceae family is vulnerable, because of the destruction of their habitat, as well as the extraction of individuals from natural populations. This is the case of the genus *Rhynchostele* Rchb.f.; among the actions considered important for appropriate conservation strategies for this genus is the generation of fundamental knowledge, such as on its reproductive biology. The objective of this work is to understand the mating system and reproductive success of *Rhynchostele cervantesii*, an endangered epiphytic orchid endemic to Mexico.

Material and methods – Manual and open-pollination treatments were conducted during 2014 and 2015 in a cloud forest in Michoacan, Mexico. In each period, 30 to 40 randomly selected inflorescences were subjected to the following treatments: a) spontaneous-self-pollination, b) emasculation, c) self-pollination, d) cross-pollination, and e) open-pollination. The developed fruits were counted and harvested, the viability of the seeds was determined, through the observation and evaluation of embryos using microscopy.

Key results – Significant differences were recorded between the treatments in both 2014 and 2015, with higher fruit production in cross-pollination than in self-pollination and natural-pollination. There were significant differences in seed viability, with higher values for seeds from open-pollination and cross-pollination and lower values for seeds from self-pollination.

Conclusions – *Rhynchostele cervantesii* is a species that requires pollinators for sexual reproduction because there is no fruit production with spontaneous-self-pollination. Under pollen limitation, the fruit set of natural pollination was a lot lower than in cross-pollination although fruits were the same quality. *R. cervantesii* had a mixed mating system with a tendency to exogamy, presenting high values of female reproductive success compared to other tropical epiphytic orchid species reported in the literature.

Keywords – Cloud forest; cross-pollination; emasculation; mating system; open-pollination; reproductive success; self-pollination; spontaneous-self-pollination.

INTRODUCTION

According to The Plant List (2013), the orchid family has 27801 species in the world, of which more than 1260 are in Mexico (Hágsater et al. 2005; Soto-Arenas & Solano-Gómez 2007). Numerous species in the Orchidaceae family are considered vulnerable around the world, because of the destruction and/or transformation of their habitat, as well as

the extraction of large numbers of individuals from natural populations (Hágsater et al. 2005; De la Torre Llorente 2018). Indeed, it is one of the best examples in which many species of one family are threatened with extinction as a result of human activities (Hágsater et al. 2005; Ávila-Díaz & Oyama 2007). This is the case of the genus *Rhynchostele* Rchb.f., which has 16 species in Mexico, seven of them being endemic (*R. aptera* (Lex.) Soto Arenas & Salazar, *R.*

candidula (Rchb.f.) Soto Arenas & Salazar, *R. cervantesii* (Lex.) Soto Arenas & Salazar, *R. ehrenbergii* (Link, Klotzsch & Otto) Soto Arenas & Salazar, *R. galeottiana* (A.Rich.) Soto Arenas & Salazar, *R. londesboroughiana* (Rchb.f.) Soto Arenas & Salazar, and *R. madrensis* (Rchb.f.) Soto Arenas & Salazar) (CONABIO 2019; Tropicos 2019). This genus mainly inhabits mesophilic mountain forest, a vegetation type that is greatly reduced in recent years, which leads to the vulnerability of this group of plants and an imminent risk of extinction (Téllez-Velasco 2011). Among the actions considered important for appropriate conservation strategies for this genus is the generation of fundamental knowledge, such as on its reproductive biology.

Plant mating systems are a combination of several factors, such as genetic compatibility, floral morphological barriers, and temporal variation in the maturation of the reproductive organs or gametes (Bawa & Beach 1981). Mating systems are considered to be influential in the levels of diversity and genetic structure of populations (Soo et al. 2001), which is an important consideration for conservation. However, the mating systems of different species can present temporal and spatial variation in orchids (Tremblay et al 2005; Ávila-Díaz & Oyama 2007) and in other species such as *Curatella americana* L. (Dilleniaceae) (Rech et al. 2018).

The great diversity of the Orchidaceae family is frequently attributed, among others, to the adaptive radiation presented by this family through the selection of specific pollinators for its cross-pollination (Tremblay et al. 2005). For example, Johnson et al. (1998) studied the patterns of adaptive radiation in *Disa* P.J.Bergius, suggesting that floral diversity in this orchid genus is the result of adaptation to pollinators. This leads to unexpected adaptations of orchids and contributes to pollinator attraction (Borba et al. 2011). Most orchid species require an external pollinating agent (Dressler 1981), among the variety of pollination systems only abiotic and mammal pollination are absent (Tremblay et al. 2005). There are also species that can reproduce without the use of a pollinator. In spontaneous autogamous systems, the flower is self-pollinated and pollination occurs in the absence of a pollinator (Haleigh & Wagner 2018).

Orchids have a high limitation of pollination. Pollen limitation found in natural plant populations with hand pollen experiments depends on historical restrictions and contemporary ecological factors (Knight et al. 2005). The factors that influence this limitation include: the abundance and distribution of pollinators, the quality and quantity of available pollen (Tremblay et al. 2005), as well as habitat fragmentation, population size decrease, resource availability, among others (Knight et al. 2005). It has also been reported that the number of flowers per inflorescence is positively correlated with the number of visits by pollinators, which can directly influence the production of fruits and the number of seeds (Sun et al. 2018).

This study will provide valuable data on the mating system and reproductive success of *Rhynchostele cervantesii* (Lex.) Soto Arenas & Salazar subsp. *cervantesii*, to suggest appropriate strategies to achieve more successful reproduction. Although there are currently populations where this species is abundant, it is considered to be an endangered species under regulatory control in Mexico (NOM-059-SEMARNAT-2010; SEMARNAT 2010), because it is widely known internationally in horticulture, and it is possible that the plants grown in other countries were extracted from their natural habitat in Mexico. Also, in some regions of the country, there is a large commercialization of wild plants during the Christmas season to decorate churches and nativity scenes. In addition, the transformation and alteration of their habitat have drastically decreased their populations in the last 20 years (Soto-Arenas & Solano-Gómez 2007). The objective of this study is to evaluate the mating system and reproductive success of the endemic orchid *R. cervantesii* subsp. *cervantesii*, through the assessment of fruit production and seed viability since more in situ propagation of this species is required.

MATERIAL AND METHODS

Study site

The sampling area is located within Área de Conservación Voluntaria de la Comunidad Indígena de Santiago Tingambato, at the Tenderio Site, which covers approximately 150 ha of the cloud forest (INEGI 2005).

Study system

Rhynchostele cervantesii is an epiphytic orchid with inflorescences with 1–6 white flowers, with slightly pink, pink, or reddish-brown concentric lines at the base of the tepals, occasionally on the lip (fig. 1A) (Espejo et al. 2002). *R. cervantesii* is an endemic and endangered epiphytic species of Mexico, distributed in the forests of the Sierra Madre Oriental, the Trans-Mexican Volcanic Belt, and part of the Sierra Madre del Sur. It is classified as threatened under Mexican law (NOM-059-SEMARNAT-2010; SEMARNAT 2010). In the study site, individuals of *R. cervantesii* are especially found on *Ternstroemia lineata* DC. and *Quercus rugosa* Née, and with a greater frequency found in the middle of the branches (Domínguez Gil 2015). The specimens of this species are:

A) MEXICO • Michoacan, Mpio. Tingambato. Predio de Tenderio belongs to Comunidad Indígena Santiago Tingambato; 19°30′30.00″N, 101°49′41.22″W; 2279 m a.s.l.; 23 Nov. 2013; fl.; *Avila-Díaz I. 2086*; AMO.

B) MEXICO • same data as preceding; 19°30′44.22″N, 101°49′52.68″W; *Avila-Díaz I. 2081*; EBUM.

Pollination treatments and fruit production

For the pollination treatments, in both years of study (2014 and 2015), it was considered to carry out 30 to 40 pollinations of each treatment such that in 2014, 91 individuals of *R. cervantesii* were pollinated (90 individuals with one inflorescence and only one individual with two, treating a total of 92 inflorescences). In 2015, 68 plants were pollinated (64 individuals with one inflorescence, three with two inflorescences and only one individual with three inflorescences, summing up to a total of 73 inflorescences).

When the buds were still closed, flowers were randomly selected and covered with thin tulle bags. Once the flowers had opened, the following treatments were applied (different flowers in the same inflorescence treated differently): a) spontaneous-self-pollination: no manual pollination was carried out, the flowers were labelled only, b) emasculation: the pollinia were removed from the flower, which was labelled in order to determine whether *R. cervantesii* produces seeds without fecundation, c) self-pollination: the pollinia were removed from the flower and placed on top of its own stigma, d) cross-pollination: the flowers were pollinated with pollinia taken from flowers of another individual, located at a distance of least 30 meters in order to minimize the possibilities of kinship, and e) open-pollination: other inflorescences were selected, labelled and left uncovered to evaluate natural pollination and female reproductive success. After applying the treatments as described in a, b, c, and d, the flowers were again covered with the tulle bags. After 12 months, the presence and number of fruits (fig. 1B) were recorded in each treatment in order to calculate the fruit set (proportion of flowers that produced fruit).

It is worth mentioning that in both years some of the inflorescences or complete plants treated were removed by people from the local community, leaving the number of treated flowers (n) as detailed in table 1.

Seed viability

In 2015, for each of the treatments, seed viability was determined immediately after opening the capsules. Observations were made with an Olympus optical microscope, Primo Star model, using the 40× objective to determine the presence/absence and characteristics of the embryo. The following categories were established: 1) empty testa, 2) seed with deformed embryo, 3) seed with elongated embryo, and 4) protocorm 1, with the globular embryo inside the testa (fig. 1C–F). It is important to state that some seeds were germinated in vitro (as in Ávila-Díaz et al. 2009), corroborating the fact that categories 1 and 2 were non-viable seeds, while categories 3 and 4 were viable. A hundred seeds from each of 15 capsules were observed under the microscope: 8 capsules from cross-pollination, 4 from self-pollination, and 3 from open-pollination.

Data analysis

To determine the differences between the treatments in terms of fruit production, a Chi square test was performed. The variation factor was pollination treatment and the response variable was the fruit production. The response variable had a binomial distribution and an associated function of Logit type was used. To determine differences in seed viability, a Chi square test was performed using the frequency data of the seed viability categories. The variation factor was pollination treatment and the response variable was the seed viability.

RESULTS

Pollination treatments and fruit production

Rhynchostele cervantesii in the study site, during the years 2014 and 2015, produced between 1 and 5 flowers per inflorescence and on average 2.75 flowers per individual. It was observed that once the flowers open, they are receptive for approximately 15 days. No floral visitors were observed.

The fruit set values were: for spontaneous-self-pollination and emasculation 0%, and for cross-pollination 54.5% and 30.8% for 2014 and 2015 respectively (table 1). Significant differences were recorded between treatments ($\chi^2 = 37.70$; d.f. = 4; p = 0.0001), taking both years into account. When each year was analysed separately, the same pattern was observed, with significant differences in the treatments of both 2014 ($\chi^2 = 28.27$; d.f. = 4; p = 0.0001) and 2015 ($\chi^2 = 19.52$; d.f. = 4; p = 0.0006), in which higher fruit production was found with cross-pollination than with self-pollination and open-pollination (table 1).

Seed viability

There were significant differences ($\chi^2 = 110.36$; d.f. = 6; p = 1.72×10^{-21}) in the seed viability categories of the fruits from different pollination treatments. The seeds from open-pollination and cross-pollination had greater viability compared to those from self-pollination (fig. 2). Raw data is provided as supplementary file (https://doi.org/10.5281/zenodo.4064918).
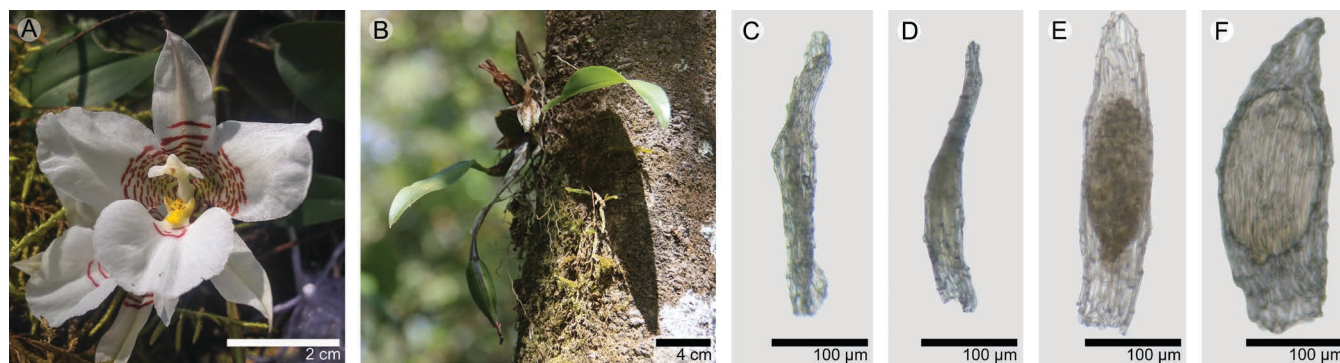
Figure 1 – **A**. Flower of *Rhynchostele cervantesii*. **B**. Fruiting *R. cervantesii* plant. **C–F**. Seed viability categories. **C**. Category 1 (empty testa, non-viable seed). **D**. Category 2 (deformed embryo, non-viable seed). **E**. Category 3 (elongated embryo, viable seed). **F**. Category 4 (globular embryo or protocorm). Photographs: A–B by Irene Ávila-Díaz; C–F by Rosa E. Magaña Lemus.

Table 1 – Production of fruits of *Rhynchostele cervantesii* in 2014 and 2015, under different pollination treatments, in the Tenderio area belonging to Indigenous Community, Santiago Tingambato, Michoacan, Mexico. Letters in superscript denote significant differences between the pairs of comparisons.

Treatment	n		Number of fruits		Fruit set (%)	
	2014	2015	2014	2015	2014	2015
Spontaneous autogamy	15	26	0	0	0	0
Emasculation	2	24	0	0	0	0
Self pollination	39	22	7	3	18[b]	14[b]
Cross pollination	33	26	18	8	54.5[a]	30.8[a]
Open pollination	59	26	8	4	13.6[b]	15.4[b]

DISCUSSION

Most orchids require external pollinating agents to achieve sexual reproduction (Dressler 1981; Tremblay et al. 2005). This is also the case for *R. cervantesii*, as no fruit production was observed with the treatment of spontaneous-self-pollination. Similar results have been observed in *Liparis makinoana* Schltr. (Soo et al. 2001), *Laelia speciosa* (Kunth) Schltr. (Ávila-Díaz & Oyama 2007), *Gomesa bifolia* (Sims) M.W.Chase & N.H.Williams (Torretta et al. 2011), *Prosthechea vespa* (Vell.) W.E.Higgins and *Cattleya luteola* Lindl. (Quiroga et al. 2010), *Prosthechea* aff. *karwinskii* (Mart.) J.M.H.Shaw (Camacho-Domínguez & Ávila-Díaz 2010), *Cuitlauzina pendula* Lex. (Pérez-Decelis et al. 2013), and representatives of the main Pleurothallidinae clades (22

species of 8 genera) (Borba et al. 2011). *Brassavola cebolleta* Rchb.f. also did not present fruit production in spontaneous self-pollination treatments or in agamospermia, indicating that it too requires pollinators for sexual reproduction, and apomixis is not observed, as it is in *R. cervantesii* (Rech et al. 2010). Some studies do report results that contrast with those of the present study, such as the ones on *Laelia undulata* (Lindl.) L.O.Williams (Quiroga et al. 2010, as "*Schomburgkia undulata*"), *Spiranthes × hongkongensis* S.Y.Hu & Barretto, *Eulophia graminea* Lindl. (Sun 1997), and *Pleurothallis ruscifolia* (Jacq.) R.Br. (CaraDonna & Ackerman 2012), which are reported to not be dependent on pollinators for sexual reproduction.

Rhynchostele cervantesii is limited by pollination since under the cross-pollination treatment, a greater amount of
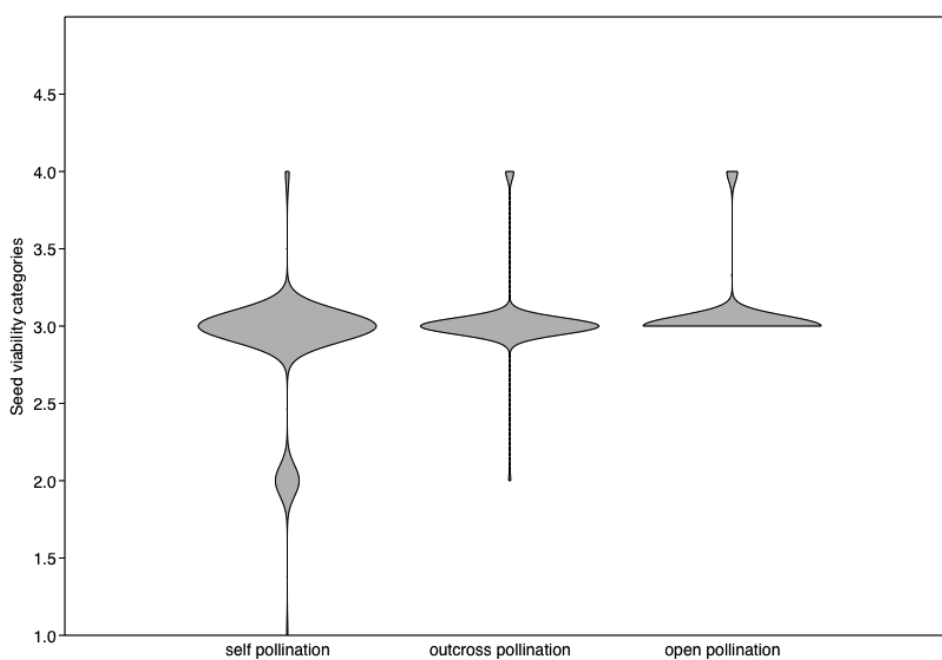
Figure 2 – Seed viability categories obtained for *Rhynchostele cervantesii* from capsules produced by different pollination treatments. 1: Empty testa (non-viable seeds), 2: Seed with deformed embryo (non-viable seeds), 3: Seed with elongated embryo (viable seeds), 4: Protocorm 1, with the globular embryo inside the testa (viable seeds).

fruit was produced in comparison to the open-pollination treatment. The species is similar in this respect to *Laelia speciosa* (Ávila-Díaz & Oyama 2007), *Prosthechea* aff. *karwinskii* (Camacho-Domínguez & Ávila-Díaz 2010), *Cuitlauzina pendula* (Pérez-Decelis et al. 2013), *Cattleya luteola*, *Xylobium variegatum* (Ruiz & Pav.) Garay & Dunst. (Quiroga et al. 2010), as well as the terrestrial species *Cypripedium bardolphianum* W.W.Sm. & Farrer, *C. tibeticum* King ex Rolfe, *C. flavum* P.F.Hunt & Summerh., *Phaius delavayi* (Finet) P.J.Cribb & Perner, and *Ponerorchis chusua* (D.Don) Soó, where species with a single flower and species with multiple flowers were compared, observing in both cases pollen limitation, recording significantly higher in species with a single flower (Sun et al. 2018). In contrast, *Brassavola cebolleta*, regardless of being pollinator-dependent, does not present pollen limitation (Rech et al. 2010). Pollen limitation may be of great importance for evolutionary or ecological processes. In particular, in our rapidly changing world, variation in the pollination environment will probably alter the population dynamics and future evolutionary potential of many plant species (Knight et al. 2005).

For the years 2014 and 2015, greater fruit production was observed in the *R. cervantesii* cross-pollination treatments, while a lower percentage was recorded in fruit production from self-pollination. Our results are similar to those obtained for *Laelia speciosa* (Ávila-Díaz & Oyama 2007), *Cattleya luteola* and *Prosthechea vespa* (Quiroga et al. 2010), and representatives of Pleurothallidinae (21 of the 22 species studied) (Borba et al. 2011), presenting high values of fruit production from cross-pollination treatments that were significantly greater than those produced by self-pollination. The ability to obtain fruits with both types of pollination (self-pollination and cross-pollination) in the same species can be an advantage to ensure the production of progeny under adverse environmental conditions or in colonization events, when availability is variable, of pollinators and/or reproductive couples (Vogler & Kalisz 2001; Kalisz et al. 2004; Haleigh & Wagner 2018). High values of exogamy as found here for *R. cervantesii*, similar to that reported for *Laelia speciosa* (Ávila-Díaz & Oyama 2007), predominate in many species pollinated by animals, considering that the unpredictability of pollinators could be a pressure of selection to obtain the ability to self-pollinate and also to have cross-pollination (Vogler & Kalisz 2001).

In contrast, the fruit production of *Laelia undulata* is reported from spontaneous-self-pollination, as well as from self-pollination, while in the natural pollination and cross-pollination treatments, no fruit production was recorded (Quiroga et al. 2010). In some terrestrial species, such as *Goodyera procera* (Ker Gawl.) Hook., a high percentage of fruit production was reported in all treatments (self-pollination, geitonogamy, and cross-pollination treatments; 92%, 94%, and 95%, respectively) (Wong & Sun 1999), and similar results are found for *Dactylorhiza maculata* (L.) Soó (Vallius 2000).

The percentage values of fruit production in the open-pollination treatment of *R. cervantesii* were 13.6% and 15.4% for 2014 and 2015 respectively. This suggests that the species has relatively high levels of female reproductive success,

compared to other tropical epiphytic orchids. These results were similar to those reported for *Comparettia falcata* Poepp. & Endl., presenting a fruit set of 17.1% in 1989, 13.1% and 12.1% in 1991, and 11.8% and 10.6% in 1992 (Ackerman et al. 1994). On the other hand, intermediate fruit set values have been reported for *Bulbophyllum humblotii* Rolfe, with values of 8% (Gamisch et al. 2014), *Trichocentrum ascendens* (Lindl.) M.W.Chase & N.H.Williams *Oncidium ascendens* Lindl. with 3.1–6.8% (Parra-Tabla et al. 2000, as "*Oncidium ascendens*"), and *Brassavola cebolleta* with 6.1% (Rech et al. 2010). For *Prosthechea* aff. *karwinskii*, a fruit set of 2.5% was recorded (Camacho-Domínguez & Ávila-Díaz 2010), while *Cuitlauzina pendula* presented a fruit set of 3.2% (Pérez-Decelis et al. 2013), and *Bulbophyllum elliotii* Rolfe presented 2% (Gamisch et al. 2014, as "*Bulbophyllum malawiense*"). For *Gomesa bifolia*, no fruit production was recorded in plants that were not subjected to manual treatment (Torretta et al. 2011). Tremblay et al. (2005) reported that the majority of orchid species present low fruit sets, however, species distributed in temperate zones present much higher fruit production than tropical species, as *R. cervantesii*.

With respect to species of terrestrial orchids, for example in *Eulophia graminea* Lindl., a relatively high value of fruit set (15%) was recorded (Sun 1997, as "*Eulophia sinensis*"), while for *Liparis kumokiri* F.Maek. fruit set values of 10.2–12.2% were recorded in the years 1999 and 2000 (Soo et al. 2001). Moreover, very high fruit set values have been reported in other species of terrestrial orchids, such as *Spiranthes × hongkongensis* and *Zeuxine strateumatica* (L.) Schltr. that each produced an approximate value of 100% (Sun 1997), while a 61% fruit set was recorded for *Dactylorhiza sambucina* (L.) Soó (Gigord et al. 2001). In contrast, low fruit set values have been reported for *Liparis makinoana*, at 0.1% and 0.2% (Soo et al. 2001).

In *R. cervantesii*, given the dependence on pollinators for sexual reproduction and the limitation of pollination, it is considered essential, within the strategies for their conservation, to include comprehensive habitat care, taking into account the complex interactions with pollinators, as is suggested by Fay et al. (2015). In this way, it is extremely important not to affect the diversity, abundance or activity of pollinators to favour a greater number of seeds and with this a probably greater recruitment.

Rhynchostele cervantesii presented greater seed viability in the fruits from open-pollination and cross-pollination treatments, while lower viability was presented in seeds from the self-pollination treatment. Similar results have been reported for the most representative species of Pleurothallidinae (22 species of 8 genera) where, in most of the species, greater viability was recorded in cross-pollination than in self-pollination treatments (Borba et al. 2011).

Considering the *R. cervantesii* seed viability values from the different pollination treatments, there was no difference between the viability from the cross-pollination and open-pollination treatments. However, when considering the established categories for *R. cervantesii* and taking the developmental stage into account, greater development was

presented (globular embryos or protocorms) in the seeds from open-pollination than in those from cross-pollination (elongated embryos, before being protocorms). This is similar to what is reported for *Laelia speciosa*, where no differences in viability evaluated by the presence of an embryo were found between seeds from open-pollination and cross-pollination, but once germinated, greater germination and development was observed from the seeds produced by open-pollination than those produced by cross-pollination (Ávila-Díaz & Oyama 2007). This differs from that reported in *Brassavola cebolleta*, where seed viability presented lower percentages in capsules from open-pollination treatments than in those from cross-pollination (Rech et al. 2010). On the other hand, *Cuitlauzina pendula* presented its highest seed viability percentages in the open-pollination treatment, followed by the cross-pollination treatment, while no viability through the presence of an embryo was recorded in the self-pollination treatment (Pérez-Decelis et al. 2013). In contrast, for the terrestrial orchid *Caladenia capillata* D.L.Jones (Peakall & Beattie 1996, as "*Caladenia tentaculata*"), no differences were recorded in the viability of seeds produced by cross-pollination and self-pollination treatments (Peakall & Beattie 1996).

With the generated data, it is considered that it is possible to increase both the production of fruits and the seed viability through cross-pollination treatments and thus enable the large-scale ex situ reproduction of *R. cervantesii* plants to make sustainable use and lower the collection pressure that is exerted on the natural populations. On the other hand, for in situ management, it is suggested to implement strategies to promote natural pollinators to protect this species and the recovery of natural populations.

SUPPLEMENTARY FILE

Supplementary file 1 – Raw data of seed viability categories of *Rhynchostele cervantesii* produced by different pollination treatments.

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