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Assessment of *in Vitro* Pollen Germination and Pollen Tube Growth of *Annona Cherimola* Mill

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ABSTRACT

Yield in cherimoya (*Annona cherimola* Mill.) is affected by various factors reducing fertilization and fruit production such as: the protogynous dichogamy of the flowers, deficient natural pollination, and low pollen quality. The objective of this study was to determine *in vitro* the differences in pollen quality of nine ecotypes and one cultivar of *A. cherimola* from pollen samples collected at the female and male phenological stages of flowering. The percentage of pollen germination and the length of pollen tubes was evaluated *in vitro* at 2 and 24 hours after incubation at 21°C, in two phenological stages of the flowers during bloom in 2018 and 2019 seasons. The germination medium contained: 10% sucrose, 1% agar, 0.005% boric acid, and 0.025% calcium nitrate. Fabulosa and Austro ecotypes and the Fino de Jete cultivar had the highest pollen germination in the male phenological stage, after 2 and 24 hours in both seasons. Fabulosa had the highest percentage of germination in the female stage in both seasons. The ideal phenological stage for pollen collection was at anther dehiscence. Austro and Fabulosa ecotypes had the longest pollen tubes after 2 and 24 hours, in both phenological stages for both seasons. Fabulosa and Austro ecotypes, and Fino de Jete cultivar, showed promising pollen characteristics for hand pollination of cherimoya.

KEYWORDS

Cherimoya; phenological stages of flowering; pollen germination; pollen tube growth

Introduction

Annona cherimola Mill. originated in Central America, with a secondary center of diversity in South America (Larranaga et al., 2017), where high genetic variability can be found, and this is one reason why the temperate valleys of Peru and Ecuador had also been considered the center of origin (Zavala et al., 2009). This species shows a great diversity of ecotypes that have adapted to a wide range of environmental conditions (Awachare et al., 2018). The selection of the best cherimoyas, by both growers and researchers led to the establishment of cultivars based on indicators that are still being defined, such as fruit quality, yield, pest and disease resistance (Gonzalez, 2013). Various cherimoya ecotypes from Ecuador have shown better fruit characteristics than varieties currently cultivated on a large scale in other countries, and could be used in breeding programs and new plantings (Scheldeman et al., 2006).

A. cherimola is the only species of the genus *Annona* adapted to the subtropical valleys of the Andean mountain range (Cautín and Agustí, 2005) and its fruit is valued for its excellent organoleptic characteristics, as well as for its nutritional and medicinal qualities (Gayoso and Chang, 2017). It is considered to be a fruit that has great potential in both national and international markets (Morales et al., 2004). This species is principally grown in Chile, Peru and Spain, with the latter being the largest producer worldwide (Cautín et al., 2010).

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The production and quality of cherimoya fruit can be affected by various factors. However, those involved in the process of pollination are, without a doubt, the most important (Santos et al., 2016). Deficient natural pollination due to a low presence of pollinating agents (Gonzalez et al., 2006), flowers with a closed structure and the marked protogynous dichogamy of the flowers (Kishore et al., 2012). Even though this specie is hermaphroditic, self-pollination is difficult because the senescence of the stigmas often occurs before the pollen grains are released (Lora, Hormaza, Herrero, 2012). The stigmas remain viable during the female stage of flowering, and when the male stage is initiated, a great number of stigmas are no longer receptive (Rosell et al., 2006).

The cycle of the flower from opening until anther dehiscence lasts for two days (Gonzalez and Cuevas, 2011); the flower opens early on the first day in the female stage and remains in this stage until the following afternoon, when the male stage begins (Lora, Testillano, Risueño, Hormaza, Herrero, 2009). This species releases mixed (bicellular and tricellular) pollen in tetrads (Lora, Herrero, Hormaza, 2009). The bicellular pollen is characterized by a longer life and a slower germination (Solís, 2012), while the tricellular pollen has a faster germination, but a more ephemeral life with greater sensitivity to dehydration, which makes its utilization more problematic (Lora, Herrero, Hormaza, 2012).

These phenomena affect both the quality and the quantity of the fruit that can be produced, necessitating the development of manual pollination techniques in order to increase yields (Lora et al., 2007). However, in various situations these techniques have shown to be inefficient (Apolonio et al., 2015). In many species, pollen quality (viability, germination capacity, and length of pollen tube growth) is affected by genetics (Calic and Radojevic, 2017), and environmental and management conditions (Prasad et al., 2011; Kuroki et al., 2017; Muradoglu et al., 2017).

Given the importance of manual pollination of cherimoya flowers in fruit production, different studies have been done on germination (Rosell et al., 1999), conservation (Pereira et al., 2014), development (Gonzalez et al., 2012) and the effects of temperature (Lora, Herrero, Hormaza, 2012); and yet, the lack of information about the cherimoya pollen quality of the local cultivars is still a problem faced by growers when they are required to manually pollinate these trees. Therefore, it is necessary to consider the existence of additional factors that can determine a successful fertilization, among them the source and quality of the pollen (Kahn et al., 1994; Pereira et al., 2014).

Thus, the objective of this study was to determine differences in pollen quality *in vitro* of the nine ecotypes and one cultivar of *A. cherimola* (cherimoya) from pollen samples collected at female and male phenological stages of flowering.

Materials and Methods

The pollen was obtained from the *A. cherimola* collection of the Austro Experimental Station of the INIAP (Instituto Nacional Autonomo de Investigaciones Agropecuarias), located in Gualaceo, Azuay province, Ecuador (02° 51' 57" S, 78° 46' 49" W) at an altitude of 2230 m above sea level. The nine ecotypes used in this experiment were: Fabulosa, CHB 027, Guille, Abu, La Loma, Tumbaco-55, La Playa, Tumbaco-61, Austro and the cultivar was Fino de Jete. These cherimoyas were originally collected from inter-Andean valleys throughout Ecuador by INIAP, and their identities correspond to either local names or to codes assigned by research technicians, with the exception of Fino de Jete, which is a cultivar from Spain. All ecotypes and cultivar were grafted onto local ecotypes. This study was done over two seasons (2018 and 2019) using 10 years old trees, grafted onto native rootstocks from *A. cherimola* seedlings and planted at 3 × 5 m.

Five individual trees of each ecotype and cultivar were selected, from which flowers were randomly collected, five in the male phenological stage (MPS) and five in the female phenological stage (FPS). The flowers were then placed in a cooler and transported to the laboratory. Closed anthers of the flowers in the female phenological stage were slightly detached from the thalamus with a sterile toothpick and placed in a Petri dish and lightly stirring with a sterile paintbrush to stimulate pollen release. In the male phenological state, the anthers were releasing pollen, therefore, the pollen was

immediately placed on the germination substrate. The pollen germination study was done in the Phytopathology laboratory of the Agronomy Career of the Catholic University of Cuenca. The germination medium consisted of 10% sucrose, 1% agar, 0.005% boric acid, and 0.025% calcium nitrate, dissolved in distilled water (Maita and Sotomayor, 2015; Rosell et al., 1999).

The medium was poured into Petri dishes (40 mm diameter) and after solidification, pollen was spread over them using a sterilized brush. The dishes were then placed in a dark incubation chamber (Memmert, Germany) with an average temperature of 21°C, and 67% relative humidity. The pollen was evaluated *in vitro* 2 and 24 hours after incubation for both phenological stages of the flowers (MPS and FPS). This study used a completely randomized design with four replications. Each experimental unit consisted of one Petri dish containing 5 ml of solid medium and pollen. The variables studied were the percentage of germination and the length of the pollen tubes. The pollen tubes were selected and measured at random, for a total of 300 tubes for each treatment, 25 for each photographed field.

Observations were done using an optical microscope (Olympus, BX41TF, Japan) with the objective lens at 10x. In each replication, three fields were photographed at random using a digital camera (Olympus, DP7, Japan, with the Image Pro Plus 7.0 program), to obtain clear images of both pollen germination and pollen tube growth. Pollen grains were considered germinated when the length of the pollen tube was equal to, or greater than, the diameter of the pollen grain (Lora et al., 2007). To calculate the percentage of germination, 100 pollen grains were randomly selected from each of the three photographs. Pollen tube length was measured in germinated pollen grains from photographs taken after 2 and 24 hours of incubation on same Petri dish. Pollen tubes were selected and measured at random, for a total of 300 tubes for each treatment, 25 for each photographed field. An analysis of variance was done for the measured variables. The percentage data were transformed into arc-sine \sqrt{x} for standardization and homogeneity of the variances. The averages were compared by the Tukey-Kramer test at 5% probability. The statistical analysis was done using the R (R-v.3.6.1, 2018) software (R Core Team, 2018).

Results and Discussion

Percentage of Germination

In the 2018 season (Table 1), the ecotype and cultivar that showed the highest percentages of pollen germination were Austro at 48% after 24 hours, and Fino de Jete at 40% after 2 hours, both with flowers collected in the MPS. While for the FPS, the highest percentages of pollen germination were Fabulosa at 32% after 2 hours; and Fino de Jete and Fabulosa at 46% after 24 hours. For MPS in 2019 (Table 2), the highest percentages were obtained by Fino de Jete at 40% after 2 hours, and Fabulosa at

Table 1. *In vitro* pollen germination and pollen tube growth of nine ecotypes and one cultivar of *Annona cherimola*, collected from flowers in female and male phenological stages during 2018 season.

	Female stage				Male stage			
	2 h		24 h		2 h		24 h	
	% ger	P. tube length (μ)	% ger	P. tube length (μ)	% ger	P. tube length (μ)	% ger	P. tube length (μ)
Fabulosa	32 a	364 g	46 a	1927 b	24 c	563 f	37 c	2102 b
CHB 027	7 h	345 h	11 f	691 j	11 g	448 h	17 g	1921 e
Guille	23 c	438 f	27 d	1444 g	9 g	504 g	30 e	1845 f
Abu	16 e	666 bc	35 c	1349 i	22 cd	793 a	23 f	1846 f
Fino de Jete	29 b	650 d	46 a	1820 c	40 a	750 b	45 b	1965 d
La loma	10 g	582 e	23 e	1670 e	21 d	667 e	32 d	1968 d
Tumbaco-55	16 e	662 cd	41 b	1713 d	14 f	729 c	21 f	2026 c
La playa	11 g	590 e	23 e	1378 h	19 e	788 a	30 e	1518 h
Tumbaco-61	14 f	677 b	25 d	1543 f	24 c	714 d	29 e	1774 g
Austro	19 d	727 a	25 d	2015 a	34 b	790 a	48 a	2273 a

ger = Germination, P = Pollen

Means followed by the same letter are not statistically different according to the Tukey-Kramer test ($p \leq 0.05$)

Table 2. *In vitro* pollen germination and pollen tube lengths of nine ecotypes and one cultivar of *Annona cherimola*, collected from flowers in female and male phenological stages, during 2019 season.

	Female stage 2 h 24 h				Male stage 2 h 24 h			
	% ger	P. tube length (μ)	% ger	P. tube length (μ)	% ger	P. tube length (μ)	% ger	P. tube length (μ)
Fabulosa	35 a	828 a	39 a	1247 d	32 c	890 a	52 a	1564 a
CHB 027	17 g	609 f	18 f	1176 e	19 f	687 d	21 h	1200 e
Guille	15 h	766 c	24 e	1081 g	17 g	803 b	28 g	1160 f
Abu	33 b	771 bc	35 b	1022 h	34 b	797 b	37 e	1179 ef
Fino de Jete	22 f	686 e	35 b	1318 b	40 a	785 b	45 b	1465 b
La loma	31 c	564 g	32 c	1138 f	24 e	685 d	33 f	1364 c
Tumbaco-55	28 d	670 e	39 a	1286 c	23 e	726 c	28 g	1338 d
La playa	15 h	609 f	17 g	1020 h	17 fg	664 d	19 h	1365 cd
Tumbaco-61	36 a	721 d	38 a	1349 a	31 cd	793 b	39 d	1384 c
Austro	24 e	789 b	28 d	1363 a	29 d	809 b	43 c	1483 b

ger = Germination, P = Pollen

Means followed by the same letter are not statistically different according to the Tukey-Kramer test ($p \leq 0.05$)

52% after 24 hours. For the FPS, Tumbaco-61 reached 36% after 2 hours, Tumbaco-55 and Fabulosa reached 39% after 24 hours. Higher quality pollen is evident in the ecotype Fabulosa and the cultivar Fino de Jete, regardless the phenological stage, with small differences at the two times that were evaluated.

These results are in line with those reported by Lora, Herrero, Hormaza, (2009) and Pereira et al. (2014), where *in vitro* pollen germination was significantly different between cultivars of *A. cherimola*. Thus, pollen quality is influenced by the genetics of the cultivars (George and Nissen, 1988; Lora, Herrero, Hormaza, 2012) and its inability to adapt to adverse environmental conditions, as demonstrated by Rosell et al. (1999), where pollen from the same variety cultivated at different altitudes showed different results.

The highest percentages of pollen germination were evident after 24 hours of incubation in the germination medium. The outstanding percentages in 2018 (Table 1) were Austro at 48% for the MPS, and Fabulosa and Fino de Jete at 46% for the FPS. For 2019 (Table 2), the best results were seen with Fabulosa at 52% for the MPS; and Tumbaco-55 and Fabulosa at 39% for the FPS. Similar results were obtained by Rosell et al. (1999) and Rosell et al. (2006), who observed a higher percentage of *in vitro* pollen germination for the cultivar Fino de Jete after 20 hours of incubation. Nevertheless, the same authors mention that once pollen becomes hydrated, whether in the stigma or *in vitro*, germination will rapidly occur and may be almost totally completed within 2 hours *in vitro* and 5 hours *in vivo*. This was also observed in some of the ecotypes used in this study, relative to *in vitro* pollen germination (Tables 1 and 2).

Additionally, the phenological stage of the flowers influenced pollen germination, with the highest percentage reached in the MPS in both seasons (Austro 48% in 2018, and Fabulosa 52% in 2019), which coincides with the works published by Pereira et al. (2014) and Bettiol et al. (2009), where higher germination was achieved with pollen collected in the male phenological stage and immediately set to germination after collection. The highest percentage of germination obtained in the FPS was related to a particular characteristic of *A. cherimola*, where mixed pollen is released at the moment of anther dehiscence (Gan and Xu, 2018; Lora et al., 2007). For this reason, the habitual practice in artificial pollination is to use pollen from flowers that are collected in pre-anthesis and then stored for about 15 hours (Gonzalez et al., 2010).

Nevertheless, the ecotype Fabulosa and the cultivar Fino de Jete (46% in 2018) and Tumbaco-55 (39% in 2019) also reached high percentages of germination in the FPS, probably due to the flowers in the female stage (day two) that were already beginning to open anthers and release pollen during collection. In this way, Lora, Herrero, Hormaza, (2012) reported that flowers collected in the female

stage passed into the male stage slightly earlier compared with female flowers that remained on the tree; this activity occurs because of the decrease in water content of the flowers which have been collected and maintained at room temperature. The pollen dehydration during collection and transport affects pollen viability and it is probably the more important reason that pollen collected in the MPS shows sometimes less germination, so that exposure and transport must be done in a short time (Nepi et al., 2001). Observations made by Alves et al. (2018), who registered high percentages of germination with FPS pollen in *A. squamosa*, were consistent with our results.

Pereira et al. (2014) evaluated *in vitro* and *in vivo* pollen germination in *A. squamosa* and *A. cherimola* x *squamosa*, and found that *in vitro* pollen germination is directly proportional to the number of fruits set in the field using the same pollen collected for the *in vitro* germination test. That is to say, the cultivars that showed the highest germination percentages *in vitro* also reached the highest fruit set in the field (Bettioli et al., 2009). The highest percentages of pollen germination in the ecotypes and cultivar of *A. cherimola* obtained in this study were comparable to the values reported for other experiments (Lora et al., 2006; Lora, Herrero, Hormaza, 2009). These results approximate the results obtained using pollen from *A. squamosa* cultivars, whose average percentage of germination varied between 40 and 54% (Kishore et al., 2012; Nietsche et al., 2009).

Pollen Tube Growth

In the 2018 season (Table 1), with pollen collected in the MPS, the ecotype that reached the greatest average pollen tube growth after 2 hours of incubation was Abu with 793 μm . Average lengths reached by Austro and La Playa not showed statistically significant differences compared to Abu; while after 24 hours of incubation, the ecotype Austro had the greatest pollen tube growth with 2273 μm . For the FPS pollen, Austro had the greatest pollen tube growth as well, with 727 μm after 2 hours, and 2015 μm after 24 hours.

In 2019 (Table 2), for the MPS pollen, Fabulosa had the greatest average pollen tube growth after 2 and 24 hours with 890 μm , and 1563 μm , respectively. For the FPS pollen, the greatest lengths were observed with Fabulosa with 828 μm after 2 hours and Austro with 1363 μm after 24 hours. Equivalent results were reported by Bettioli et al. (2009) in *A. cherimola*, where they observed an average pollen tube length of 600 μm after one hour of incubation.

The pollen tube growth observed in this study were the longest reported to date for *A. cherimola* after a 24 hours incubation period. Lora et al. (2018) reported a maximum length of 450 μm after 9 hours of incubation. This could be due to the fact that the pollen was collected just before anther dehiscence, stored at 4°C and used the following day. Bettioli et al. (2009) utilized fresh pollen and registered pollen tube lengths of 600 μm only one hour after it was placed on the growth medium, while pollen that has been stored began germination only after 6 hours of incubation (Alves et al., 2016).

The phenological stages of the cherimoya flowers influence the pollen tube growth, with the best results observed for flowers collected in the MPS. The variations observed in pollen tube growth for one single ecotype in the two seasons could be related to the prevailing environmental conditions during each season, as mentioned by Alves et al. (2018), who registered different percentages of germination and pollen tube growth in dry or rainy seasons for *A. squamosa*.

Fabulosa and Austro ecotypes and Fino de Jete cultivar had the highest pollen germination in the male phenological stage, after 2 and 24 hours of *in vitro* incubation during the 2018 and 2019. Meanwhile, Fabulosa had the highest pollen germination from flowers collected in the female phenological stage after 2 and 24 hours of incubation for both seasons. Male phenological stage had the highest quality pollen, showing higher pollen germination and greater pollen tube growth. Of the nine cherimoya ecotypes studied, Austro and Fabulosa had the greatest pollen tube growth, in both phenological stages of flowering, during both seasons.

From the evaluation of the germination percentages and pollen tube growth of nine ecotypes and one cultivar of *A. cherimola* from the *ex situ* collection of the Austro Experimental Station of the Instituto Nacional Autónomo de Investigaciones Agropecuarias (INIAP), it was determined that there are significant differences in pollen quality. Fabulosa and Austro ecotypes and cultivar Fino de Jete showed promising characteristics for use in manual pollination in commercial cherimoya orchards. So, it is necessary to evaluate the pollen of the best ecotypes found in this research in commercial orchards to improve the yield of cherimoya.

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Disclosure Statement

No potential conflict of interest was reported by the authors.

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