Article

Viability and longevity of pollen in Mexican lemon genotypes estimated by *in vitro* germination

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Abstract

Studies of pollen viability and longevity in the genotypes selected as progenitors are useful to ensure the success of hybridizations in a breeding program. The present study was carried out in the Tecoman Experimental Field of INIFAP, in Tecoman Colima, Mexico, during the months of January and February of 2008 and 2009. The viability and longevity of the pollen were estimated in three varieties of Mexican lemon [*Citrus aurantifolia*): ‘Colimex’, ‘Colimon’ and ‘Lise’, two natural hybrids of Mexican lemon; CV 63-64 and CV 67-68 as well as the ‘Citrange C-35’ (*C. sinensis* x *Poncirus trifoliata*) as a control. The formulations of Brewbaker and Kwack (1963); Lora *et al*. (2006); Leal (1969) modified were evaluated. The three culture media promoted *in vitro* germination of pollen grains in very similar proportions, close to 20%. The genotypes, ‘Colimon’ and ‘Lise’ registered low germination percentages of 0.78% and 3.05% respectively. On the other hand, the variety ‘Colimex’ and the natural hybrid CV 63-64, had intermediate percentage values, while the ‘Citrange C-35’ and the natural hybrid CV 67-68 reached the highest pollen viability percentages (43.3% and 39.69% respectively) and can function as pollen donors. The pollen of all genotypes evaluated, conserved at room temperature, which fluctuated between 21 °C and 30 °C, lost almost 100% of its viability 48 h after anthesis. This is the first study on the viability and longevity of Mexican lemon pollen using *in vitro* germination tests.

Keywords: *Citrus aurantifolia*, pollen germination, pollen longevity, pollen viability.

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Introduction

The sexual or conventional hybridization is one of the methods of genetic improvement of plants that poses the opportunity to recombine genomes and create genetic variability in the new combinations of genes contributed by the parents involved in the crosses. Hybridization works usually begin with the identification of the desirable characteristics of the parents within the available germplasm, among which the viability and longevity of the pollen in the progenitors is an indispensable requirement (González *et al.,* 2002; Soares *et al.,* 2013; Olatunji *et al*., 2016).

The quality of pollen is a fundamental parameter for studies related to the biology of pollination, since there are numerous essential processes for genetic improvement and the production of crops that depend on this parameter (Rejón *et al*., 2010). Therefore, for the breeders the pollen quality is fundamental since it allows them to assure the success of the pollinations in their hybridization programs (Sulusoglu and Cavusoglu, 2014; Olatunji *et al*., 2016). Another reason for its value lies in the efficiency it provides for the production of fruit with good characteristics for the consumer, since it affects the volume of fruit setting (Sharafi, 2011) and when producing seeds it increases the synthesis of gibberellins resulting in greater size and quality of the fruit (Zhang *et al*., 2010).

The physiology of pollen, especially germination and viability, has received considerable attention to be applied, both in programs of plant breeding, as in the conservation, adaptation and understanding of the physiological behavior of the fertilization of pollen grains (Khan and Perveen, 2014). The characterization and viability of pollen grains are useful tools to guide crosses in breeding programs (Soares *et al*., 2013), since it allows to discriminate and use only the best pollen donors and to make good use of it in pollination works (Gaaliche *et al*., 2013; Baswal *et al*., 2015).

The viability of pollen varies substantially between and within species and can be reduced by different factors (Yeamans *et al*., 2014). According to these authors, pollen becomes unfeasible either during its development in flower buds or after its full development. On the other hand, Davarynejad *et al*. (2008), indicate that the formation of fertile pollen depends on environmental factors such as humidity and temperature and genetic factors such as morphology, capacity for germination and growth of the pollen tube. Environmental factors such as high and low temperatures, relative humidity and water stress cause a reduction in metabolism and a short life cycle (Khan *et al*., 2013; Khan and Perveen, 2014).

The viability of pollen and its germinative power in the laboratory can be determined by a): *In vitro* germination tests (Moura *et al*., 2015; Shekari *et al*., 2016); b) measurement of enzymatic activity (Gozlekci *et al*., 2011; Soares *et al*., 2013; Demir *et al*., 2015); and c) the cytoplasm staining of the pollen grain (Maiti and Rodríguez, 2015, Baswal *et al*., 2015). However, Soares *et al*. (2013); Kundu *et al*. (2014); Shekari *et al*. (2016), agree that *in vitro* germination is more reliable since staining methods can overestimate the viability of pollen. On the other hand, the longevity of pollen, considered as the period of time during which pollen maintains its viability, that is, the capacity for germination and fertilization, varies greatly with plant species and storage conditions (Dafni and Firmage, 2000).

Studies have been carried out to determine the viability and longevity of citrus pollen (Khan and Perveen, 2014; Kundu *et al*., 2014; Baswal *et al*., 2015; Demir *et al*., 2015; Ahmed *et al*., 2017); however, there are no known studies conducted on Mexican lemons, so their behavior regarding these pollen characteristics is unknown at this time. Therefore, this research was carried out with the purpose of determining the viability and longevity of the pollen of three varieties of Mexican lemon, two natural hybrids and the 'Citrange C-35' as a control, which are part of the germplasm bank of citrus fruits from the Tecoman Experimental Field of INIFAP and which are used in the Mexican lemon genetic improvement program.

Materials and methods

The study was carried out in the Biotechnology laboratory of the INIFAP Campo Experimental Tecoman in the months of January and February of 2008 and 2009. Trees of six to eight years of age were used. The collection of the flowers was carried out between 9:00 and 10:00 in the morning, the hour in which the highest frequency of opening of flowers in Mexican lemon occurs and the dehiscence of the anthers begins (Robles-González and Medina-Urrutia, 1984).

Culture media for *in vitro* germination

Media for *in vitro* germination were prepared according to the formulations of Brewbaker and Kwack (1963) (BBK): $CaNO_3$ 300 mg L^{-1}, $MgSO_47H_2O$ 200 mg L^{-1}, H_3BO_3 100 mg L^{-1}, KNO_3 100 mg L^{-1}, Sucrose 100 mg L^{-1}. Lora *et al*. (2006): $MgSO_47H_2O$ 200 mg L^{-1}, Ca $(NO_3)_24H_2O$ 250 mg L^{-1}, KNO_3 100 mg L^{-1}, H_3BO_3 100 mg L^{-1}, Sucrose 80 g L^{-1}. Leal, (1969) modified: sucrose 80 g L^{-1}. The pH was adjusted to 6.5 for the three media, gelled with 10 g L^{-1} agar and sterilized in a vertical AESA brand autoclave for 20 min at 121 °C and under pressure of 15 lb $inch^{-2}$. Reactive grade mineral salts of the SIGMA brand were used.

Estimating the viability of newly released pollen from anthers

For this study, fresh pollen of the varieties 'Colimex', 'Lise', 'Colimon', the natural hybrids of Mexican lemons CV 63-64 and CV 67-68 and the 'Citrange C-35' as a control, extracted two hours after anthesis, were used. 25 to 30 newly opened flowers with anthers in the process of pollen release were collected. The flowers were placed in sterile plastic Petri dishes of 10 x 100 mm and two hours later they were rubbed together to encourage the pollen to come off the open anthers. For pollen sowing, the drop method was used on the slide. With the help of a micropipette, five drops of the culture medium were deposited on the slides. Five slides were prepared for each culture medium.

Once gelled and cold drops, the pollen grains were dropped on them with the help of a brush of camel hair bristles. The slides were labeled and placed in a humid chamber to avoid dehydration of the culture medium. The wet chamber was taken to the incubation room for a period of 24 h with a temperature that fluctuated between 25 and 28 °C. After that time the samples were observed with the 40x objective of a Motic BA200 compound microscope. In each drop five fields were observed and in each the total number of grains visualized was quantified, as well as the number of grains that emitted and developed their pollen tube, as indicated (Kalinganire *et al*., 2000). With these data, germination percentages were determined.

Determination of the longevity of pollen preserved at room temperature

For this experiment we used pollen from the diploid genotypes 'Colimex', 'Colimon' and 'Lise', as well as 'Citrange C-35' as a control. 25 to 30 newly opened flowers with anthers releasing pollen were collected. The flowers were placed in Petri dishes and rubbed together to encourage the pollen to come off the open anthers. After obtaining the pollen, it was kept in the Petri dishes and kept at room temperature of the laboratory, which fluctuated between 21 and 30 °C, until its use for the viability tests, which were carried out 2, 24 and 48 h after the anthesis. For the sowing, observation and recording of variables the methodology described above was used.

Analysis of data

In both experiments, an experimental randomized block design with a factorial arrangement and five repetitions was used. To estimate the viability of fresh pollen, three culture media and six genotypes were tested. To estimate pollen longevity, three culture media and four genotypes were used. The analysis was made with the averages of two years of study. The percentage values were transformed to arcsine values by means of the equation: Bliss [$y = \arcsin(\sqrt{(x/100)})$]. Andres *et al*. (1999). The statistical analyzes were carried out with the help of statistical software Statistix 9 from Analytical Software, (2010).

Results and discussion

Estimating the viability of newly released pollen from anthers

The three culture media allowed the germination of pollen grains of the different genotypes studied (Figure 1). After 24 h, growth of the pollen tube could be observed from the pollen grains, which were quantified as viable. The results recorded in 2008 coincided with those obtained in 2009 for both culture media and genotypes.

Figure 1. Germination of Mexican lemon pollen grains 'Colimex' in three culture media a) Brewbaker and Kwack (1963); b) Lora *et al*. (2007) modified; and c) Leal (1969).

For the statistical analysis, the averages of the two years of study, of each of the registered variables, were used. The Anova did not report significant differences for the means of cultivation factor. However, highly significant differences ($p= 0.01$) were detected for the genotype factor, as well as for the interaction between culture media∗genotypes.

According to the results shown in Table 1, it can be seen that the three culture media evaluated promoted the germination of pollen grains in very similar proportions and were statistically equal to each other. The percentages of germination in the three culture media fluctuated between 20.4% to 21.6%.

Table 1. Percentage of pollen grains germinated in five Mexican lemon genotypes and the 'Citrange C-35' in three culture media.

Genotypes	Culture medium			Averages by genotypes
	Brewbaker and Kwack (1963)	Lora *et al.* (2007)	Leal (1969)	
'Colimon'	1.04 fg	0.18 g	1.11 fg	0.78 e
'Lise'	4.33 fg	3.37 f	1.46 fg	3.05 d
'Colimex'	13.94 de	16.9 de	10.4 e	13. 74 c
CV 63-64[1]	22.23 cd	21.45 cd	30.02 bc	24.57 b
CV 67-68[1]	45.12 a	40.45 ab	33.49 abcs	39.69 a
'Citrange C-35'	42.95 ab	41.17 ab	45.95 a	43.35 a
Mean average	21.6 a	20.59 a	20.4 a	

CV= 14.71. Averages with different letter within groups are statistically different (Tukey $p< 0.05$). [1]= natural hybrids of Mexican lemon.

According to Soares *et al*. (2008) *in vitro* germination is one of the methods commonly used to estimate the quality of pollen grains, in studies of reproductive biology and selection of male parents in breeding programs. It is the main indicator of the functional viability of pollen (Cerovic *et al*., 2014). To this end, simple and some more complex culture media have been developed that aim to reproduce the conditions provided by the stigma of the flower so that the pollen germinates, and the pollen tube grows in style, until reaching the embryo sac.

In our study, no differences were found in the germination percentages of germinated pollen grains among the three culture media evaluated. From the simplest formulation (Leal, 1969) modified, which only included 80 g L^{-1} of sucrose + 10 g L^{-1} of agar, in which an average of 20.4% of germinated pollen grains was reached, to the middle more complex (Lora *et al*., 2006), which in addition to carrying 80 g L^{-1} of sucrose and 10 g L^{-1} of agar, added some mineral salts and averaged 21.6% of germinated grains.

This means that the pollen newly released from the anthers in the six genotypes studied, does not require a complex culture medium and as indicated by Patel and Mankad (2014); Ahmed *et al*. (2017) a satisfactory germination of pollen can be achieved in a solution of water and sugar. Therefore, any of the three formulations evaluated is adequate to estimate the viability of Mexican lemon pollen in the laboratory. If we take into account the composition of the three media, the one described by Leal (1969) and modified in this study, it is adequate for the simple and inexpensive preparation.

The study also allowed to detect differences among the six genotypes evaluated with respect to the ability of pollen to germinate *in vitro*. Based on the results, the three varieties of Mexican lemon presented low percentages of germination with respect to the other genotypes evaluated. Particularly the varieties ‘Colimon’ and ‘Lise’ registered germination percentages of 0.78% and 3.05% respectively, so it is considered that these genotypes are not desirable as pollen donors in breeding work.

On the other hand, the variety ‘Colimex’ reached 13.74% of germinated grains, although it remains a low percentage of viable pollen compared to that reported by Khan and Perveen (2014); Ahmed *et al*. (2017) for other citrus fruits, can be used as a male parent with better expectations of fertilizing the ovule and generating hybrids.

The result obtained for ‘Colimex’ coincides with that reported by Viloria and Grosser (2005) who recorded 7.5% viable pollen, although these authors made their estimates using the 1% acetocarmine staining method. On the other hand, the ‘Citrange C-35’ reached the highest pollen viability percentages and can therefore be considered as a good candidate to be used as a pollen donor (Table 1). According to these results, the ability of *in vitro* germination of pollen was different between Mexican lemon varieties and between citrus species. (Domínguez *et al*., 1999; Kundu *et al*., 2014; Khan and Perven 2014; Ahmed *et al*., 2017) also reported differences in pollen germination percentages among different citrus species.

Determination of the longevity of pollen preserved at room temperature

In this experiment the Anova reported highly significant differences for the storage time factor after anthesis (TDA), for the genotypes factor and for the interaction TDA∗genotypes. According to the results shown in Table 2, it is clear that treatments with pollen stored at room temperature for 2 and 24 hours from the anthesis showed values between 10.03 and 13.92% of pollen grains with the ability to germinate and they were statistically equal to each other. In the case of pollen preserved for 48 hours in a natural environment before sowing, it practically lost its viability. On the other hand, the genotypes presented a different behavior for this variable and recorded different percentages of germinated pollen grains, which made them statistically different from each other. The ‘Citrange C-35’, reached the highest percentage of pollen germinated *in vitro*, followed by ‘Colimex’, ‘Lise’ and ‘Colimon’.

Table 2. Percentage of pollen grains germinated in three Mexican lemon genotypes and the ‘Citrange C-35’ and three storage times.

Hours after anthesis	Genotype				Average per hour
	‘Colimón’	‘Lise’	‘Colimex’	‘Citrange C-35’	
2	0.63 f	3.78 e	12.71 c	38.57 a	13.92 a
24	2.17 e	8.53 cd	7.64 d	21.77 b	10.03 a
48	0.16 f	0.46 f	0.17 f	0.34 f	0.28 b
Average genotype	0.98 d	4.25 c	6.84 b	20.23 a	

CV= 29.94. Averages with different letters within groups are statistically different (Tukey $p < 0.05$).

It can be seen that the genotypes show interaction only with treatments of 2 and 24 h of storage. With the pollen planted two hours after the anthesis, the genotypes reached different percentages of germination, being statistically different from each other. The 'Citrange C-35' presents the highest percentage of germination, followed by the variety 'Colimex', 'Lise' and 'Colimon'. With pollen planted after 24 h from the anthesis, the genotypes showed a behavior similar to that described for the treatment two hours after anthesis. For treatment with pollen stored for 48 h, the germination percentages of pollen grains were significantly reduced in all genotypes.

The longevity of pollen grains is an important characteristic for a breeding program through conventional hybridization. The duration of viability, monitored over time and by recording the ability of pollen to germinate *in vitro*, can help estimate its longevity. In many plant species this period of time is usually short if the pollen is maintained at room temperature, although according to Dafni and Firmage (2000), this can vary strongly between species and storage conditions.

In this regard, Wang *et al*. (2012) determined that under conditions of cloudy days, the pollen of fodder grass (*Festuca arundinacea* Schreb.) transgenic and nontransgenic, remained viable up to 240 min, with 5% viability after 150 min of storage. However, under a sunny environment, the viability of pollen is reduced to 5% in only 30 min, with a complete loss of viability in 90 min.

There is little information detected regarding the longevity of citrus pollen. However, Khan and Perveen (2014), point out that in storage under refrigeration conditions (4 °C), the pollen of *C. limon*, *C. aurantium*, *C. reticulata* and *C. sinensis* lost about 50% of their capacity to germinate *in vitro* compared to that observed in fresh pollen, after 48 weeks, while *C. paradisi* lost about 95% of its viability in that same period of time. These authors did not evaluate the viability with stored pollen at room temperature.

On the other hand, Ahmed *et al*. (2017), in a similar work, determined that the pollen of three grapefruit varieties (*C. paradisi*), on average, lost 80% capacity to germinate *in vitro*, compared to that registered with fresh pollen, after 48 weeks of storage at 4 °C, whereas in two varieties of Tangelo (*C. reticulata* × *C. maxima* × *C. paradisi*), three of grapefruit (*C. maxima*) and three of mandarin (*C. reticulata*) the loss was 60% and in three varieties of orange, 49% was lost. In that same study, pollen samples of all species and varieties conserved at room temperature did not present viable pollen grains after 8 days.

In our study, the pollen was kept in the laboratory at room temperature, which fluctuated between 21 °C and 30 °C, which caused the pollen germination capacity to be rapidly reduced. The pollen samples taken two hours after the anthesis reached the highest percentages of germination in the genotypes 'Colimex' and the 'Citrange C-35'. In the samples taken 24 h after the anthesis the germination percentages were almost halved in both genotypes. While in the genotypes 'Colimon' and 'Lise' the results were slightly different, and the highest percentages of germination were reached at 24 h after the anthesis. However, for 48 h of storage, viability was significantly reduced in all evaluated genotypes, which registered on average 0.28% of germinated pollen grains.

A similar result was described by Shekari *et al*. (2016) in nettle border (*Leonurus cardiaca*) who recorded germination percentages of 82.84, 24.37, 11.04 and 0.19 in pollen samples collected at 2, 24, 48 and 72 h after anthesis, while pollen stored at -60 °C maintained the highest germination values *in vitro* after 48 weeks of storage. It is necessary to continue this work and determine the best conditions for conservation at low temperatures or cryoconservation, which will help to have pollen at any time of the year and match seed donors and improve the genetic improvement of Mexican lime.

Conclusions

The viability of Mexican lemon pollen of the 'Colimex' and 'Lise' varieties was low compared to what was observed with the 'Citrange C-35', but it can be useful to be used as a male parent in breeding programs for hybridization. The longevity of pollen from Mexican lemon genotypes and 'Citrange C-35' is only 24 hours if stored at room temperature, therefore, if pollen is conserved under this condition, it should be used the same day of release of the anthers or maximum 24 h later to obtain better results of fertilization of ovules.

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