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Viability of maize pollen grains *in vitro* collected at different times of the day

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The viability of pollen grains is the essential precondition for obtaining enhanced or hybrid vigor genotypes and a good fixation of the fruit. It is a matter of great importance, especially for genetic improvement programs, which are used in various types of controlled pollination. This study aimed to evaluate the viability of the maize pollen grain through in vitro germination and stainability tests, collected at different times. The experimental design was a randomized block with factorial 2x5, two days of pollination at five times (9:00 a.m., 11:00 a.m., 1:00 p.m., 3:00 p.m. and 5:00 p.m.) with four replications. Each treatment consisted of 12 plants, which were taken randomly within each plot. The parameters evaluated were: germination percentage, the percentage of pollen grain stainability, the stigma receptivity and the best time for pollination. Through the analysis of variance, it was noted and interaction between the days and times of collection and highly significant differences for the following parameters: temperature percentage, humidity, germination and viability of the pollen grain, indicating that the days and times influenced the viability of pollen grains. We could observe that the best results of viable pollen grains were obtained at 09.00 a.m. regardless of the day. It was also noted that the ambient temperature and relative humidity were the main influencing factors on pollen viability, and not the collection times.

Key words: Genetic improvement, pollination, *Zea mays* L.

INTRODUCTION

The viability of pollen grains is essential precondition for obtaining enhanced or hybrid vigor genotypes and a good fixation of the fruit. It is a matter of great importance, especially for genetic improvement programs, which are used in various types of controlled pollination (Borém and Miranda, 2007).

The release of pollen grains can start from sunrise until noon, depending on the temperature, humidity and

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genetic constitution of the plant. Under natural conditions, the maize pollen grain does not have a large strength and can lose viability within a range of one to four hours after being released into the atmosphere (Ferreira et al., 2007).

Various techniques are used to assess the viability of pollen grains, the most common being: germination *in vivo* and *in vitro*, besides the chemical dyes test, which is based on cytological criteria such as coloration (Almeida et al., 2011).

The germination *in vitro*, in culture medium, is a technique that emulates the style-stigma conditions, inducing germination of the pollen tube. Each species requires a specific formulation of culture medium to obtain good germination of the pollen grain. Among the elements that compose the culture medium, sucrose is considered essential, while the boron as boric acid, and calcium, as calcium dihydrate can maximize the medium efficiency. The agar is used to give consistency to the medium and avoid damage to the pollen tube during the evaluation (Ferreira et al., 2007).

The stainability is a quite simple procedure, inexpensive and provides results quickly, making it very attractive for works involving pollen grains. Considering that there is a correlation between viability and stainability, the estimation is given by counting the aborted and not aborted pollens showing stained and unstained, respectively (Alvim, 2008). Various dyes may be employed for this purpose, highlighting: acetic carmine, triphenyltetrazolium chloride, aniline blue and malachite green with acid fuchsin.

The viability of the pollen grain is not only influenced by intrinsic factors such as its state of physiological maturation, origin, genetic characteristics, the plant nutrition and by extrinsic factors such as the composition of the culture medium, pollen density in the medium, temperature and incubation time, collection period, but also by environmental conditions such as temperature, humidity, etc. (Stanley and Linskens, 1974).

Almeida et al. (2002) reported that *in vitro* germination of pollen grain is influenced by environmental conditions such as temperature and relative humidity during the collection and the maturing phase of the tassel, as newly formed gametes are more viable than pollen grains matured. The same authors also mention that the *in vitro* germination of pollen is highly correlated with fertilization in the field, in other words, *in vivo*. However, fertilization tends to be smaller than *in vitro* germination due to the influence of factors such as stigma receptivity, genetic barriers, temperature and relative humidity.

Considering these facts, it is necessary to raise and provide data about the feasibility of maize pollen grains, because it is a preliminary and essential condition for the success of the hybridization in the genetic improvement programs. The obtained results can contribute to future works, which aim at the storage of the pollen grain, besides enabling the crossed pollination between genotypes without reproductive synchrony, facilitating the search for superior individuals with greater genetic purity (Ferreira et al., 2007).

Few are the papers relating the influence of different times of collection in the feasibility of maize pollen grains, enhancing the importance of papers like these in improvement programs, aiming at better results as for pollination. In the light of the foregoing, this study aimed to evaluate the viability of the maize pollen grain through *in vitro* germination tests and the stainability, collected at different times.

MATERIALS AND METHODS

The experiment was conducted in the harvest of 2013/2014, in the experimental farm and Biotechnology Laboratory of Pontifícia Universidade Católica do Paraná, Campus Toledo, located in the city of Toledo, western Paraná 24°43'48 "S, 53°44" 24 "W, in an altitude of 560 m. Based on Köeppen classification, the climate is Cfa, mesothermal humid subtropical, with hot summers with a tendency of rainfall concentration, with an average temperature above 22°C. Winters with little frequent frosts with temperatures below 18°C, without a defined dry season. The average rainfall in the region is 1800 mm per year (Rubel and Kottek, 2010). The precipitation and air temperature, which occurred during the crop cycle can be observed in Figure 1. The soil used in this study was classified as typical Eutroferric Red Latosol, with smooth and wavy terrain and clayey texture (Embrapa, 2012).

The experimental design was a randomized block with factorial 2x5, two days of pollination at five times (9:00 a.m., 11:00 a.m., 1:00 p.m., 3:00 p.m. and 5:00 p.m.) with four replications. The size of each plot was 3 m long and 3.6 m wide, totaling 10.8 m², with 521.64 m² of total plot area. We used a population of four plants per meter, totaling 42 plants per plot. Each plot consisted of four rows of maize, in which the sample was done with 12 plants from the central area (useful area), discarding 1 m on each side of the plot.

During the work, it was necessary to perform some crop practices such as soil analysis, covering fertilization, weed and pests control and protection of male and female inflorescences. We conducted a chemical analysis of soil and subsequent correction with 240 kg ha^{-1} of 8-20-20 NPK formulation. The seeds of CD384Hx maize hybrids were treated with imidacloprid at 0.25 L dose ha^{-1} and seeded on October 11, 2013. Fertilization in coverage was performed with 80 kg ha^{-1} of urea in the V6 stage of maize. The weed control was done by hand weeding at 14, 30 and 70 DAS (days after sowing). The application of physiological insecticide was made with Teflubenzuron 0.1 L ha^{-1} at 17; 26 and 40 DAS and contact insecticide Methomyl 0.4 L ha^{-1} at 40 DAS.

Once observed the emission of female inflorescence, they were covered with plastic bags to prevent contamination. Later, about 70 DAS, when the tassels had viable pollen, these were covered with paper bags so that it could be preceded with the pollen collection, and all the bags were informed with date. After seven days, when it was obtained a representative number of the sampling, we selected the plants with recently packaged pollen, and the tassels were beaten separately within each paper bag and made a bulk of pollen of the cultivar, which was conducted to the Biotechnology Laboratory of PUC-PR to carry out the *in vitro* tests.

Germination test

The pollen grains were incubated in culture medium, which was

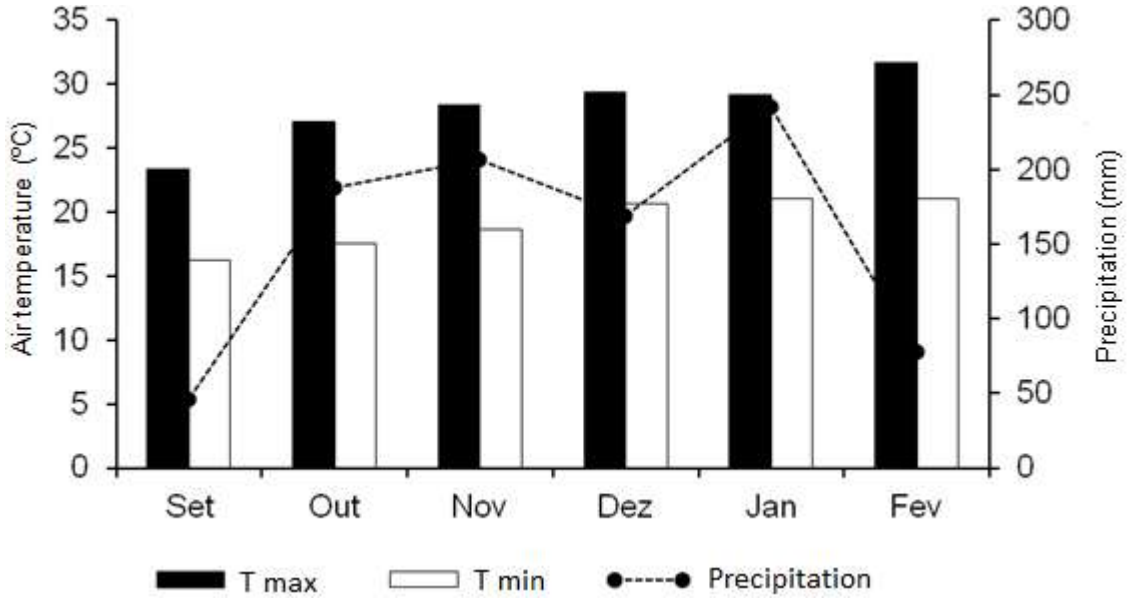

Figure 1. Representative graphic of maximum temperature, minimum and precipitation during the crop cycle by the Climatological station at Universidade Católica do Paraná - Campus Toledo. Toledo – PR, 2015.

determined prior to conducting the research (values are not shown). The culture medium used to determine the germination percentage was composed of 15% Sucrose; Boric Acid 0.01%; Calcium Nitrate 0.025%; 0.6% Agar, with pH 6.0. The incubation temperature was 25°C in a Biochemical Oxygen Demand (BOD). After two hours of incubation, the number of germinated grains was counted. The germinated pollen grains count was done with the aid of optical microscope with a 10x objective increase, evaluating four fields of view, corresponding to four repetitions. In each field of view there were on average 40 pollen grains. They were considered germinated, grains that had pollen tubes which exceeded the length of the diameter of the pollen grain itself (Pio, 2004).

Stainability test

For the determination of this parameter, we used the dye 2,3,5 triphenyltetrazolium (TTC) at 1%. A pollen sample was spread over a glass slide and then added a dye drop, closing the set with a cover slip. The observations of the number of viable and non-viable pollen were performed two hours after the preparation of the slides. The counting of viable and non-viable grains was made following the same germination procedure, in which it was considered viable pollen grains (red color) and non-viable (brown color) (Dafni, 1992).

Stigma receptivity

It was determined with the aid of a magnifying glass through observation of air bubbles formation when depositing hydrogen peroxide (H_2O_2) at 3% on the stigma surface, according to Dafni (1992). While performing the collection of pollen grains, temperature and humidity readings of air through the digital device Portable Digital Thermo-Hegrometro were carried out. The parameters used in the experiment were: percentage of germination, the percentage of stainability of the pollen grain and the stigma receptivity. The data were submitted to analysis of variance (ANOVA) and when detected significant effects between treatments, the regression test was performed at a level of 5% probability, with the help of statistical program Costat 6.4 (COHORT SOFTWARE, 2003).

RESULTS AND DISCUSSION

Through the analysis of variance, it was found the existence of highly significant differences between the days and times of collection and the interaction between them, for the following parameters: temperature percentage, humidity, germination and stainability of the pollen grain, indicating that collection times were differently affected by days. Regarding the blocks there was no significant difference (Table 1).

Maize is a plant which is relatively tolerant to water stress during the growing season, but shows extreme sensitivity, with a decrease in grain yield, if there is no water in the stages of flowering and grain filling (Bergamashi et al., 2004). In this context, an important variable in estimating the water consumption of a culture is its evapotranspiration (ETc), which according to Doorenbos and Kassam (1994), is dependent on the knowledge of the reference evapotranspiration (ETo), with regard to weather conditions the site of interest, along with the physiological and morphological characteristics of the culture, represented by its culture coefficient (Kc), which incorporates plant characteristics (such as leaf area index) and effects evaporation of soil, varying over the cycle depending on the growth rate and, hence, the variation of the ground cover (Allen et al., 1998).

A large number of methods, with greater or lesser degree of empiricism, are being developed over the past

Table 1. Analysis of variance for the parameters temperature (°C); humidity (%); germination (%); viability (%) of maize pollen grain with *in vitro* cultivar CD 384Hx, Toledo, PR, 2015.

Fatores	Temperature (ºC)	Humidity (%)	Germination (%)	Viability (%)
Day	0.001*	0.001*	0.001*	0.001*
Hours	0.001*	0.001*	0.001*	0.001*
Day x Hours	0.001*	0.001*	0.001*	0.001*
Block	0.853^{ns}	0.253^{ns}	0.191^{ns}	0.982^{ns}
Average	31.8	55.9	14.6	30.6
C.V. (%)	1.0	2.1	24.4	17.8

ns, *: Non- significant, significant at 5% probability, respectively, by Regression test.

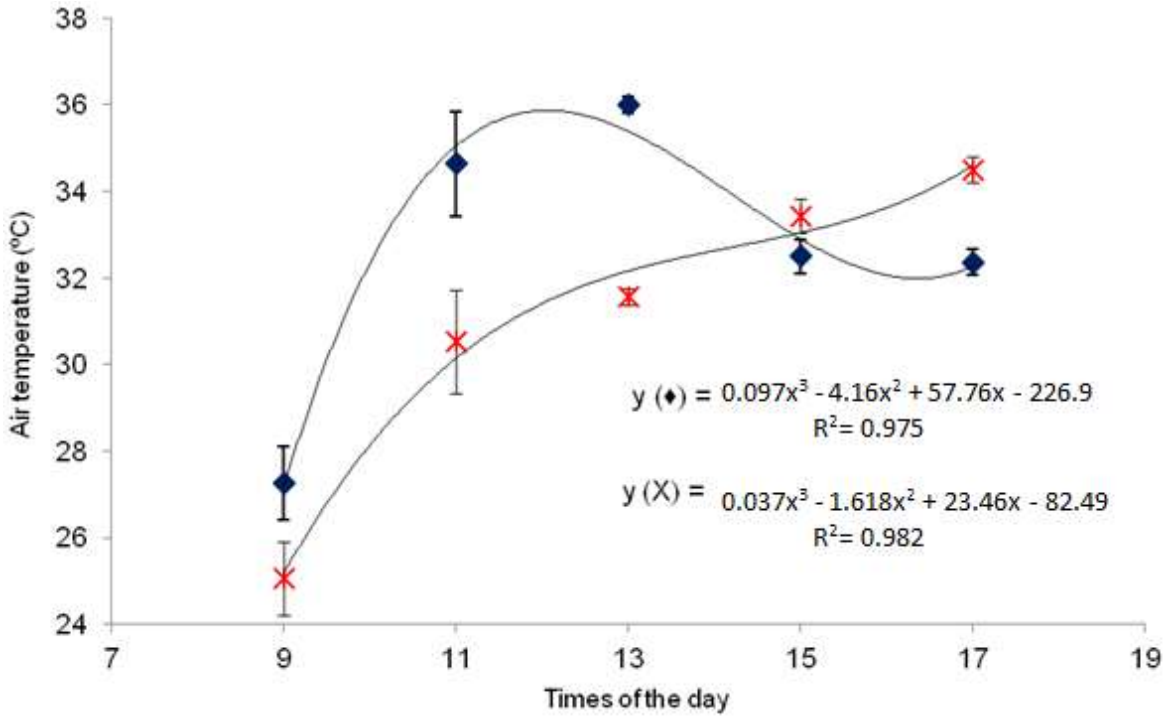

Figure 2. Air temperature (° C) of the two days of maize pollination in the field, day 1 (♦) and day 2 (X), and their respective time differences between each evaluation day. Toledo - PR, 2015.

decades in an attempt to estimate the evapotranspiration from different climatic variables (Valipour, 2014 a, b, d, e, f, g; 2015). The proposed correlations are often successfully tested in local calibrations, however, they are not universally applicable. To solve this problem, FAO has promoted studies to evaluate the available methods in order to obtain a standard method for calculating the ETo. After extensive testing, the Penman-Monteith method with the parameter proposed by the FAO came to be recommended as the standard method used in the daily scale. This is the method with the highest probability of correct answers in a wide variety of locations and climates (Allen et al., 1998). In this way, the most important weather parameters are temperature, relative humidity, and wind speed for evapotranspiration models.

By analyzing the air temperature, it was found that there was a significant difference between the two days at all times of collection evaluated (Figure 2). On day 1, the lowest temperature was at 9:00 a.m. (27.2°C), with its peak at 1: p.m. (36.2°C), decreasing gradually in the following times. Regarding the second day, the lowest temperature was also in the first collection time at 9:00 a.m. (25.3°C). The temperature rose gradually in the following times, peaking at 5:00 p.m. (34.5°C). This difference in temperature between the days is due to the fact that the first day of collection was preceded by about seven days without rain, which contributed to the rise in

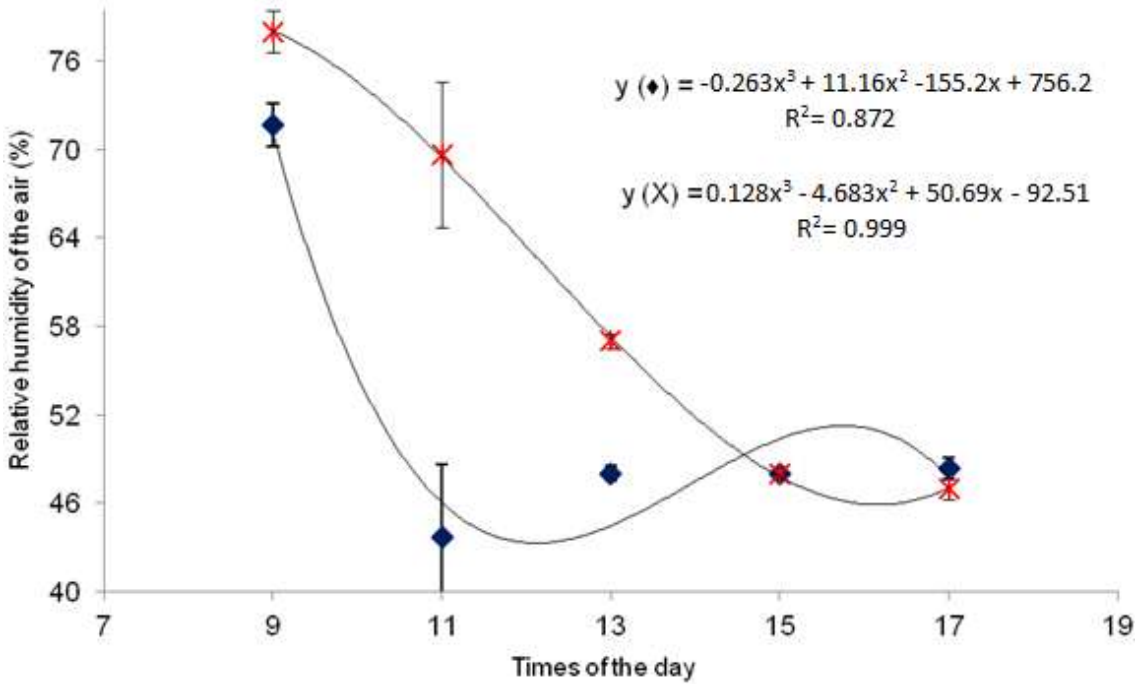

Figure 3. Relative humidity of the air (%) of the two maize pollination days in the field, day 1 (♦) and day 2 (X), and their respective differences between each evaluation time of the day. Toledo - PR, 2015.

temperature. It should also be borne in mind that one day before the collection on day 2, there was a shower of rainfall, increasing the relative humidity and consequently reducing the temperature.

Sorghum, being a C4 plant as maize, has its optimum range of air temperature during the vegetative period between 26 to 34°C (Hammer et al., 1993); and during the reproductive period between 25 to 28°C (Prasad et al., 2006, 2008). Decreases in pollen viability and consequently fewer pollen grains are a result of high temperature stress during pre-anthesis (sporogenesis), resulting in decreased seed set in sorghum grain (Prasad et al., 2008). High temperature stress during floret development alters pollen morphology and results in an abnormal exine wall, degeneration of tapetum cells, and membrane damage, leading to pollen sterility in grain sorghum (Djanaguiraman et al., 2014), wheat (Prasad and Djanaguiraman, 2014), and soybean (Djanaguiraman et al., 2013a,b).

The effect of thermal stress during reproductive development has been further investigated in tomato. Sato et al. (2002) reported that temperatures between 20 to 25°C are ideal for tomato. Surprisingly, increasing temperature to 29°C dramatically decreased the number of fruits and seeds formed. When evaluating the influence of the pollination temperature in the acquisition of haploid embryos in intergeneric cross wheat x maize, Silva et al. (2002) reported that 20 to 30°C temperature are optimal for obtaining embryos.

The relative air humidity differed significantly in two days and in all collection times, except for the fourth time (Figure 3). On day 1, the highest percentage of humidity was obtained at 9:00 a.m., with 72% RH, while the lowest percentage (44% RH) was observed at 11:00 a.m., with a small increase to 1:00 p.m. (48% RH), maintaining the same humidity in the following times. On day 2, the higher humidity was also at 9:00 a.m. (78% RH), reducing gradually the following times up to 5 h to 47% RH, which had lower results. This is due to the fact that humidity is correlated to the temperature, being inversely proportional behavior, and thus, these results may be explained in the same manner as above.

The effect of ambient relative humidity on pollen viability is evident in many investigated species. The response of high or low humidity, however, may differ among species and is generally associated with the intrinsic hydration state of the pollen in dehiscence (Nepi et al., 2001).

According to Aylor (2004), the maize pollen grain when exposed to dehydration by ambient conditions, can lose substantially all viability within three hours, as measured by *in vitro* germination of pollen grains. The author further states that the exact time of viability depended on the humidity during the experiment, that is, 20% RH, for example, the entire viability was lost in 50 min, whereas 75% RH pollen still existed after four hours.

Fonseca and Westgate (2005) found that after one hour in an environment of approximately 32°C and 50% RH, maize pollen grain loses approximately half of its water content (from 60 to 28.9%). For the same period of time, but 28.5°C temperature and 85% RH, the pollen

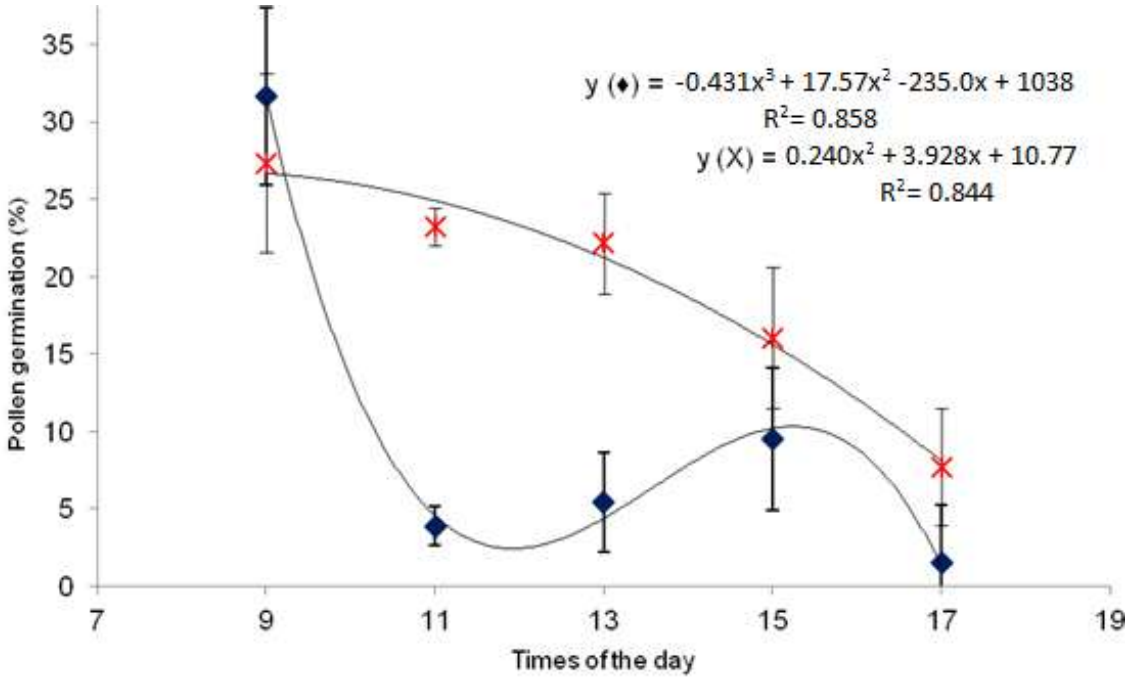

Figure 4. Maize pollen grains germination with *in vitro* using medium of germination (%) at different times and days of pollination in the field, day 1 (♦) and day 2 (X), and their respective differences between each evaluation time of the day. Toledo - PR, 2015.

grain water content remained around 54%. These values are relevant since the functional pollen life has a close relationship with its water content, and this turns out to be influenced by temperature and relative humidity.

For the germination of pollen grains we observed a significant difference between the two days and collection times (Figure 4). On day 1, the highest percentage of germination was observed at 9.00 (32%), and this time the relative humidity was 72% and the ambient temperature of 27.2°C. At the following times there was a great swing and decreased to 11.00 (4.8%) and rising to 13 and 15:00 and again, declining 17.00 (2%), obtaining lower germination. On day 2, the highest percentage of germination was also at 9.00 (27%), but unlike the first day, gradually declined to the 17.00 (9%), with the lowest percentage of germination. This shows that the percentage of pollen grain germination is directly related to the relative humidity, and conversely, to the ambient temperature.

For the germination percentage of pollen grains we observed a significant difference between the two days and collection times (Figure 4). On day 1, the highest percentage of germination was observed at 9:00 a.m. (32%), and at this time the relative humidity was 72% and the ambient temperature of 27.2°C. At the following times there was a great oscillation, having a decrease at 11:00 a.m. (4.8%) and rising at 1 and 3:00 p.m. and again, declining at 5:00 p.m. (2%), obtaining lower germination. On day 2, the highest percentage of germination was also at 9:00 a.m. (27%), but unlike the first day, it gradually declined to 5:00 p.m. (9%), with the lowest percentage of germination. This shows that the percentage of pollen grain germination is directly related to the relative humidity, and conversely, to the ambient temperature.

Corroborating the results obtained, Almeida et al. (2002) reported that *in vitro* germination of pollen grains is influenced by environmental conditions such as temperature and relative humidity during the collection and the maturing of the tassel, as newly formed gametes are more viable than matured pollen grains.

Scorza and Sherman (1995) consider that a good pollen must present 50 to 80% of sprouted grains with well-developed tubes, and with the aging of pollen grains, the percentage of germination and the length of pollen tubes decrease. This explains the germination percentages not reaching higher rates because the protections of the banners were held for five days in a row until they reach 12 plants with viable banners.

Results of this work are evidenced by Alvim (2008), studying the feasibility and conservation of maize pollen grains. In his study, evaluating the best time to collect maize pollen grains (9:00 am, 2:00 and 4:00 p.m.) achieved the best results at 9:00 a.m., with 60% germination. At this time, the relative humidity was 80% and the ambient temperature of 19.5°C.

Costa et al. (2012) when studying the effect of collection time on the viability of maize pollen, reported

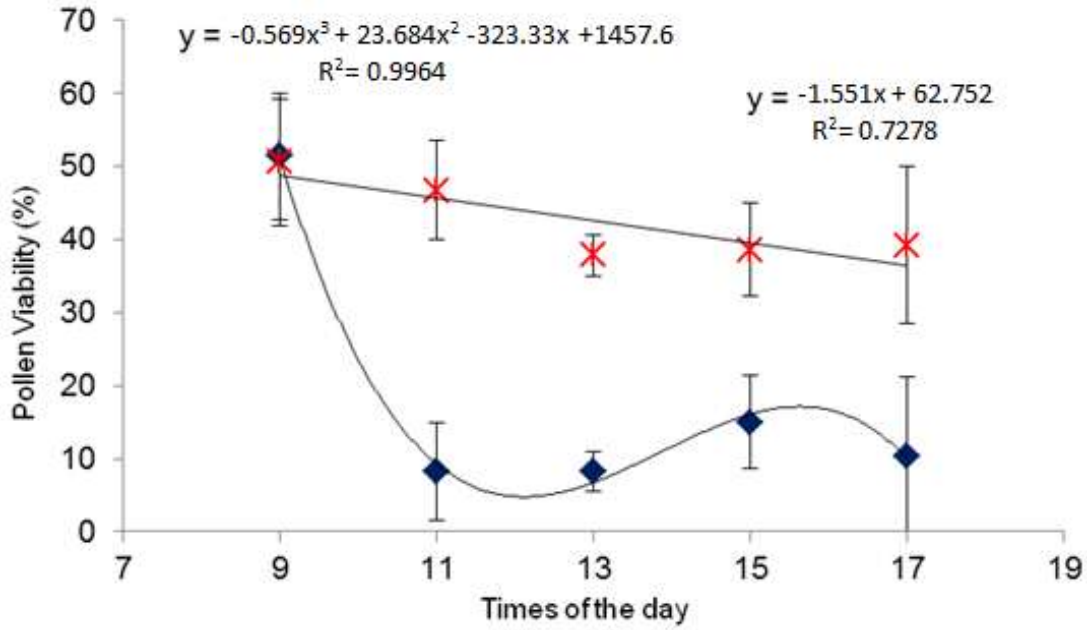

Figure 5. Viability of maize pollen grains with *in vitro* using Stainability Test (%) at different times of the day and pollination in field, day 1 (♦) and day 2 (X), and their respective differences between each evaluation time of the day. Toledo - PR, 2015.

that regardless of the cultivar, the first hours of release of maize pollen grains occur in the morning (8:00 to 12:00 a.m.), being more suitable for collection by providing higher germination values. Likewise, Maeda et al. (2012) in his work on *in vitro* assessment of the viability of maize pollen, found the best results at 10:00 a.m. for most crops during germination and in the viability test.

Figure 5 shows the analysis of the viability of maize pollen at different times of collection and days, and it can be seen that the cultivar is influenced by the collection times and the days of anthesis. The first collection time (9:00 a.m.) showed the best results 50.5 and 50% on days 1 and 2, respectively. The lowest viability results there were 12 and 35%, at 5:00 to 1:00 p.m. for days 1 and 2, respectively. The viability follows the germination curve, but always with higher values, showing the relationship between them.

The use of dyes is a very attractive technique, especially by the ease and speed in obtaining results (Alvim, 2008). However, the validity of this method has been questioned by the difficulty of distinguishing the color of viable pollen grains or immature and aborted pollen grains. For the realization of this method, trained and keen eye people are needed to realize these differences. Under favorable conditions, the pollen grain can remain viable for up to 24 h. Its longevity, however, can be reduced when subjected to low humidity and high temperatures (Aylor, 2002). In maize, it was found that the pollen grains do not support a humidity reduction higher than 50% without loss of its normal functions, with the proper humidity content of around 20% (Ferreira et al., 2007).

According to Ferreira et al. (2007) when studying conservation and determining the viability of maize pollen grains, noted that the study of three different collection times (9: 00 a.m., 2: 00 and 4:00 p.m.) showed that the highest percentage of germination in vitro, consequently the viability, was at 9:00 a.m., with about 60%.

Regarding the style-stigma receptivity, there was 100% receptivity, showing that all plants were able to receive pollen grain in all the times. According to Ritchie and Hanway (1989), the establishment of direct contact between pollen grain and viscous pile of the stigma stimulates the germination of the first, originating a structure called pollen tube, which is responsible for the ovule fecundation inserted in the ear. Fertilization occurs in 12 to 36 h after pollination, a period which is variable depending on some factors involved in the process such as water content, temperature, contact point and style-stigma length.

Fanceli and Dourado (2000) also cite that the environmental stress in this stage, specially the hydric one, causes lower pollination and lower grain formation of the ear, once under drought, both the “hair” and the pollen grains tends to desiccation. Therefore, the number of fertilized ovule is closely related to the nutritional status of the plant, with the temperature as well as humidity condition of the soil and air. In this way, knowing all the factors that influence the viability of maize pollen grain is extremely important, as this is the main genetic material

used in plant breeding programs.

Conclusion

It was observed that the best results of viable pollen grains were obtained at 09:00 a.m. regardless of the day. It can be noted that the ambient temperature and relative humidity are the main factors of influence on pollen viability, not the times of the day.

Conflict of Interests

The authors have not declared any conflict of interests.

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