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Physiological characterization of some male pollinators in Tunisia and study of the effect of conditioning temperature on the viability and germination of pollen

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Abstract – In Tunisia, the cultivation of the date palm is the pivot of the agricultural activity of the regions of South West and particularly in the areas of Djerid and Nefzaoua. It constitutes the framework of the oases which are unique sources of greenery and life in the middle of the desert. The cultivators of the date palm were able to deduce the importance of the origin of the pollen used on the quantity and the quality of the production of the dates. Consequently, they use selective pollinating male varieties. The importance of male palms, locally named "Dokkars" on the quality and quantity of dates has been the subject of numerous works. In Tunisia, this genetic potential of the date palm, unrecognized wealth is now threatened, which makes it necessary to prospect and study this phylogenetic resource in order to protect it by the selection of better feet. Our present work, which is part of this framework, concerns the study of thirty samples of pollen harvested from the palm groves of the region of Djérid. In a first step, a characterization of the physiological parameters of the pollen was carried out, namely the germination test, viability, pH, conductivity and water content. In the second part, we worked only on ten pollinators. We tried to evaluate the effect of pollen conditioning temperature on the two main fertility parameters (germination and viability) by selecting two temperatures: room temperature and 4 ° C and this over a period of 8 months. The results obtained showed: the existence of variability in physiological characteristics from one individual to another, which reflects the great diversity of the Dokkars; The existence of a negative correlation between germination rate and pollen conductivity. Finally, the use of the refrigerator at 4 ° C, allows a better conservation of viability and germination of pollen.

Keywords: Dokkars, pollen, viability, germination, pH, water content, conductivity, refrigeration.

1. Introduction

The date palm (Phoenix dactylifera L.) is a symbolic tree of arid regions. This tropical species, dioecious, monocotyledonous is well adapted to hot and dry Saharan climates. It is also one of the oldest cultivated plant species. It is of great interest because of its high productivity, the nutritive quality of its highly sought-after fruits and its ability to adapt to the Saharan regions. Indeed, the date palm is a perennial species, with a slow growth, it does not enter production until several years. In addition, it is dioecious, the male and female inflorescences are borne by separate individuals and that only the female plants produce fruit (dates).

Tunisia holds 25% of world sales, and is the fifth country in the world from the harvesting point of view, as it produces 9% of the world's production of dates (GIF. 2015). At the national level, the total production of dates is positively changing over time. These statistics predict a harvest of dates of about 69600 tons in 2000-2001, compared with 241,666tones in 2016/2017. It should be noted that date exports during the 2016-2017 season (until 30 September 2017) amounted to about 109.8 thousand tons worth nearly 569.3 million dinars (MD) against 110 thousand dinars tons for the amount of 473.7 MD during the same period of the 2015-2016 season. Tunisian dates have been exported to nearly 80 countries, according to GIF (inter-professional fruit

grouping). Date and quantity production is intimately linked to pollinators because the quality of pollen is a determining factor in yield (Kadri et al 2015). Pollen plays a crucial role in the expression of certain characteristics of the fruit. It is therefore necessary to carry out studies using morphological and physiological markers in order to detect the differences that may exist between the different types of pollen in order to distinguish good pollinators for farmers. Date production in quantity and quality is intimately related to pollinators 'dokkars' because the quality of the pollen grain is a determinant of yield. (DARLEEN et al., 1988) mentions that pollen influences not only the size and shape of the seeds (xenia) but also the size, shape, weight and rate of maturation of the fruit (metaxenia). As a result, it appears that pollen plays a significant role in the expression of certain characteristics of the fruit. It is therefore necessary to carry out studies in order to detect the differences that may exist between the different types of pollen, in order to draw up a list of good quality dokkars (Sedra.2013). However, surveys and research conducted over several years nationwide were mainly oriented towards female palms, and secondarily on male palms (Dokkars). In this context, a number of studies were carried out between 1985 and 1994 by Dr. Larbi Boughdiri (Professor at the University of Annaba) on pollens from the Biskra region and contributed to a better understanding of the criteria, to characterize the quality of date pollen and their use in distinguishing between different male palms. This work aims to characterize on a physiological level a collection of pollinators of date palm in order to distinguish the pollinators by their physiological criteria. Another part will be devoted to the study of the effect of temperature on pollen conservation.

2. Materials and methods

2.1. Plant material

The study was carried out on a collection of 30 date palm pollinators, these feet were coded by the letter P followed by a number or a name, the sampling was carried out on two sites: the first is located in Tozeur (South West Tunisia) in the experimental plot of the Regional Research Center of Oasis Agriculture (CRRAO) and which includes pollinators: P164, P159, P92, P27, P25, P113, P157, P36, P126, P160, P167 78, P40, P101, P24, P146, P161, P61, P20, P39, P59. The second is in the CRRAO plot in Dgueche: P6, P7, P10, P8, P4, P9, P2, P Sami and P In Vitro.

2.2. Pollen sampling and conservation

The experiment was carried out on a large male inflorescence, harvested on 01 March, in order to limit the variability of the plant material. The inflorescences of a palm tree are not necessarily of the same size nor of the same quality. Indeed, the time of emission and flowering influence the quantity and quality of the pollen. The inflorescence was harvested from a male foot aged 45 years, producing between 30 and 45 spathes annually. At the time of extraction, male flowers should ideally be exposed to a temperature of 30-32 ° C in a dry atmosphere. Alcaraz et al. (2012) and Webber (1996) recommends exposing male flowers to temperatures of 25 to 30 ° C in an atmosphere with a relative humidity of less than 40%. If the pollen is not extracted quickly, the small amount of reserves contained in each grain will be quickly used, respiration will be impaired, and pollen will be killed quickly (Dutta et al. 2013). After sifting the pollen and separating it from the waste from the flowers, it is weighed. The pollen is then placed in kraft papers at room temperature and the pollen stored at +4 ° C in glass boxes each bearing a label which indicates the number of the pollinator and the weight of the pollen harvested (Sedra.2013).

2.3.Physiological study

2.3.1. Germination test

Ching and Ching (1964) consider it sufficient that the length of the pollen tube is greater than or equal to the diameter of the grain. For Pfeiffer (1955), the length of the tube must be greater than three times the diameter of the grain. For Christiansen (1969), only pollen grains that develop pollen tubes where the presence of reproductive cells can be observed are considered germinated. The in vitro germination test allows knowing the capacities of the pollen to germinate in an artificial environment without interaction with the stigmata of the flowers; this medium is that of Brewbaker and Kwack (1963), modified (BKM). The seeding of the pollens is carried out under a laminar flow hood. The grains of pollen are spread evenly inside the petri dish containing the Brewbaker and Kwack medium modified by a 50-micron sieve. Each box has the name of the pollinator as well as the temperature of the conservation. Incubation is carried out for 24 hours in an oven set at 27 ° C. Pollen counts are carried out using an optical microscope at a magnification 40 linked to the computer screen. The result is the average of three replicates for each pollen. The percentage of germination is determined by the following ratio:

Germination rate = (Number of germinated seeds / Total number of grains observed) * 100

2.3.2. Viability test of pollen grains

This test is based on the property of certain dyes to react in the presence of organic molecules. The concentration of these molecules will determine the intensity of the staining that will indicate the state of maturation of the pollen grain (Stanly and Linskens 1974). Kaplay (1991) tested other dyes, such as acetic carmine, which reveals the presence of genetic material. This test is based on microscopic observation at a magnification of 40 X of the pollen grains which rely between slide and cover slip and stained left dried for 15 minutes. The viable pollen grains are colored red while the dead grains are not colored and have a wrinkled appearance. The counting is carried out by observing 3 microscopic fields.

The rate of viability is expressed by the ratio:

Viability rate = (Number of viable grains / Total number of grains observed) * 100

2.3.3. Measurement of conductivity

Ching and Ching (1976) developed a technique to measure the conductivity of a pollen filtrate. This filtrate is obtained as pollen grain ages after it is immersed in a given volume of deionized water for a specified period of time. The observed conductivity is the consequence of the release of ionized substances in the reaction medium. A high conductivity translates a massive release of compounds from the cell. This leaching is attributed to poor quality of cell membranes unable to retain cellular metabolites. This technique consists in preparing a quantity of pollen of 30 mg in a volume of 10 ml of water and then incubation was carried out for 16 hours in an oven set at 27 ° C. (Ching and Ching 1976; Carlos et.2016). The conductivity is then measured by a conductivity meter (COND6 +). Conductivity measurement is simple to perform and gives fast results. This is why it has been used by several authors to evaluate the viability of pollen, especially during the storage of the samples (Foster and Fbridgwater 1979, Wbber 1987, Phlippe et al., 1991, Webber and Bonnet. 1993).

2.3.4. pH measurement

Charpentier and Bonnet (1983) showed that the best percentages of pollen germination are obtained with pH between 4.3 and 6.1. The technique for determining pollen pH is to prepare a mixture of 0.25g of pollen in 3ml of distilled water in tubes. These components are mixed by a stirrer and then introduce the electrode of the pH meter previously calibrated (Kemilla et al.,2016).

2.3.5. Water content of pollen

The water content of the pollen plays a predominant role in maintaining the viability of the pollen during its conservation. Reducing the water content of a pollen grain greatly reduces its respiration (Hoekstra and Bruinsma1975). The water content corresponds to the amount of water contained in the pollen. It is presented in three forms: the first corresponds to the vital water contained in cytoplasm structures (free water). This is involved in the maintenance of turgor and in most biochemical reactions (Bouafia. 1996). The second, the water contained in the membranes (bound water). From this depends the activity of biological molecules (Bouafia. 1996). The third represents the water bound to the macromolecules and the wall (Kehroas 1986; Boughediri, 1994). The water content is expressed in terms of the weight of the fresh matter (FWM) in percent; Obtained after desiccation of a fresh pollen quantity (0.5 g) of each sample for 48 hours in an oven set at 110 ° C; Then weighed to determine dry weight (DWM). A second weighing after calcinations at 250 ° C. for 4 hours makes it possible to determine the total water content. The water content is calculated according to the following formula:

Rate water content = FWM-(DWM / FWM)

2.3.6. Effect of temperature on physiological parameters

The experiments were carried out on a batch of 5 pollen samples selected from the first batch of 30. The choice of these 5 samples was based on the morphological variability existing between these cultivars (Adra.2013; Alcaraz et al.,2011). In fact these five feet present the maximum of phenotypic diversity existing in this collection. The tests were carried out on the same samples of pollen but kept in two different temperatures an ambient temperature ranging from 25 to 28 ° C and a regenerating temperature of 4 ° C. Measurements of viability and germination parameters were carried out at the beginning of each month for a period of 10 months from May 2016 to February 2017.

2.3.7. Statistical analysis of data: Analysis of variance

Each experiment was repeated 5 times, the results were subjected to analysis of variance ANOVA using statistical software STATISTICA. The comparison of means and the establishment of order classes (homogeneous classes) are made by the Newman-Keuls test at 5% probability level.

3. Results and Discussions

3.1.Germination rate

In Vitro germination tests give a fairly rapid assessment of the quality of pollen samples (Moody and Jett 1990). Unlike cytochemical tests, in vitro germination requires the ability of the pollen to emit a pollen tube, which is the mechanism that occurs in nature at a crossing. These tests require only a very small amount of pollen (less than 0.1 mg). Figure 1 illustrates the pollen germination test of the pollinators studied. The germination rates were obtained by microscopic observation with a magnification of (40 x), this germination test was carried out on fresh pollen. Statistical analysis of this variable showed significant differences between pollen germination rates. The percentage of "in vitro" germination of the pollens varies from 42, 72 to 97,78 %.

Through these results, with reference to PEYRON (2000), which considers that pollen must germinate in vitro to more than 60% in order to ensure good fruit set. We can distinguish three classes of qualities:

- Poor: whose rate is less than 42%, formed solely by P Sami.
- Medium: with rates between 50% and 72%, formed by P 164; P27; P25; P167; P146; P161 and P9.
- Good: with rates ranging from 80% to 97% formed by the rest of the samples.

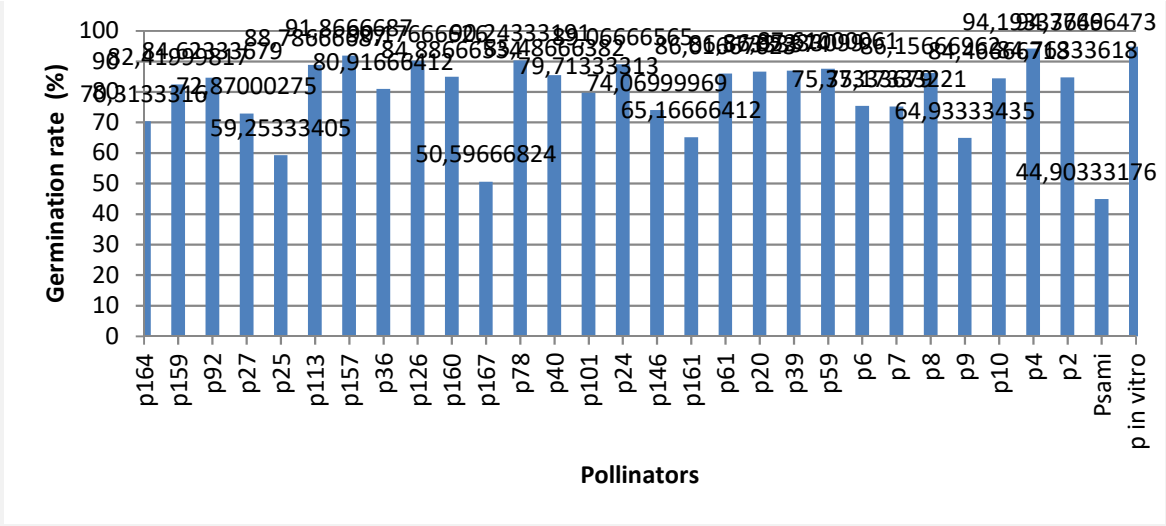

Figure 1: Variation of the initial germination rate of pollen as a function of pollinator

Storage conditions should be optimized to maintain both Viability and vigor (Shivanna et al., 1991). The in vitro germination test can be used to evaluate the pollen's vigor, provided that the percentage of germination is measured over time and compared to fresh pollen reactions (Shivanna et al., 1991). Brewbakerand Kwack (1963) showed that the concentration of pollen on the culture medium had an influence on the percentage of germination observed. We note that more the density was higher more the development of the pollen tubes is favored. Colas and Mercier (1994a) obtained similar results with birch pollen as well asAhlgren and Ahlgren (1978) with white pine pollen. This phenomenon has the consequence of producing variable results if one does not take into account the density of seeding. It is therefore very important to carry out in vitro germination tests, to use constant conditions of seeding density. The interpretation of a germination test should be done with caution. A low percentage of germination is often attributed to poor pollen quality. Germination rates vary greatly between species. In addition, the needs of the same pollen sample may vary after the one under stress such as conservation (Polito and Luza 1988).

3.2.Study of viability

The viability test was carried out on fresh pollen. Figure 2 illustrates the various pollen viability percentages of the pollinators. The statistical analysis of this variable also showed significant differences between pollen viability rates. Indeed, the majority of pollen have shown rates of high viability and almost equal. The highest level was obtained with the pollinator P157 (99.33%) followed by the pollinator P113 (98.57%) and P164 (97.47%). The lowest rate was obtained with pollinators P126 (82.78%) followed by P7 (77. 12%).Achieving colorimetric tests to assess the viability of pollen batches can quickly get results. Alami et al. (1988) carried out a study of the effects of cold on the viability of sorghum pollen. These authors used four cytochemical tests to evaluate the viability of their samples. The results are very variable from one test to another. For their part, Binder et al. (1974) analyzed different staining methods and concluded that these techniques are not satisfactory in determining the viability of pollen.

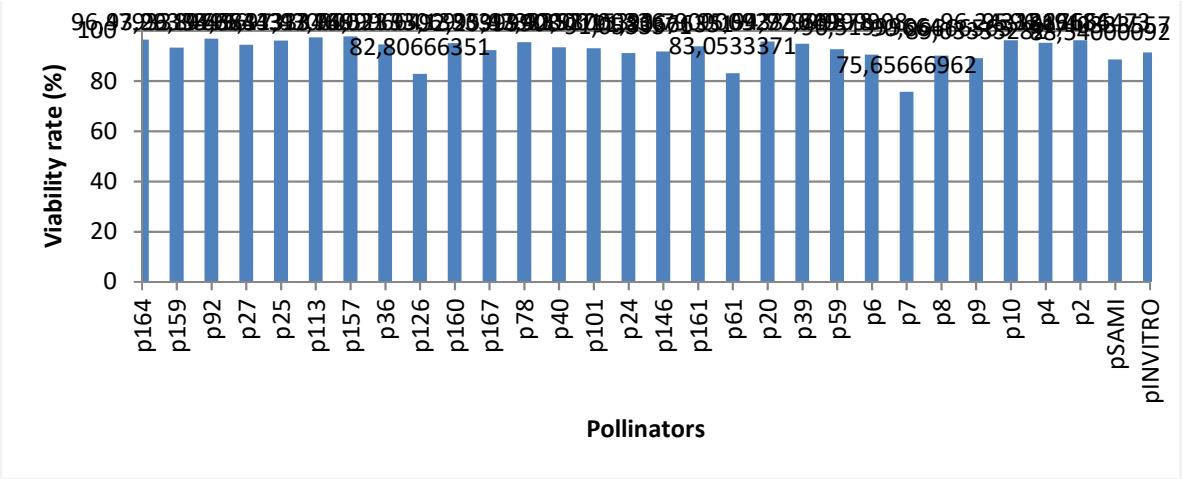

Figure 2. Variation of the initial viability rate of pollen based on the pollinator

In fact, these staining depend not only on viability, but also on temperature, duration of exposure and species. Moreover, the results are not only expressed in terms of living or dead. An intermediate range can represent different degrees of vigor and degeneration. Thus, during an experiment, it is preferable to produce several colorations rather than one in order to obtain a more reliable result. These methods are accurate for identifying pollen grains free of cytoplasm or without enzymatic content, which are unable to germinate. However, due to the presence of grains that cannot be classified as live or dead due to their intermediate staining, other tests have been developed to increase the reliability of the results; Infrared spectroscopy testing can also illustrate the changes Biochemical processes in pollen grains during germination (Sowa and Conner 1995).

3.3.pH measurement

The pH values of the pollens studied range from 6.95 (P7) to 6.29 (P39). These values are very close (Figure 3). To our knowledge, this factor has not been addressed in advance. The present attempt not only aims to quantify this factor, but also to determine the relationship that may exist with the germinative power of "in vitro" pollens. In this context, a correlation test was performed by Halimi (2004) and whose correlation coefficient r = -0.3337. It thus indicates a very weak negative correlation.

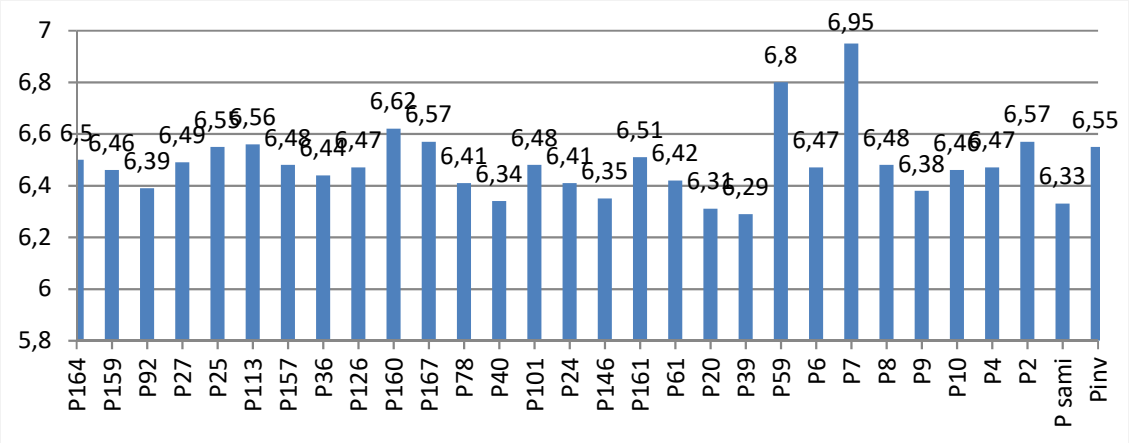

Figure 3. Variation of the pH value of pollen based on the pollinator

3.4.Conductivity Test

Conductivity measurement is simple to perform and gives fast results (Carlos et al., 2016; Alcaraz et al. 2011). This is why it has been used by several authors to evaluate the viability of pollen, especially during the conservation of the samples (Foster and Bridgwater 1979, Webber 1987, Philippe et al., 1991, Webber and Bonnet-Masimbrt 1993; Geitmann and Palanivelu. 2007). The conductivity measurements are used to get an idea about the quality of pollen, more the conductivity value is higher, more the quality of the pollen is bad (Carlos et al.,2016; Metez et al.,2000). From Figure 4, the highest conductivity was observed in P 4 and P Sami pollinators (0.38). The lowest level was observed in pollinators P113 (0.203).

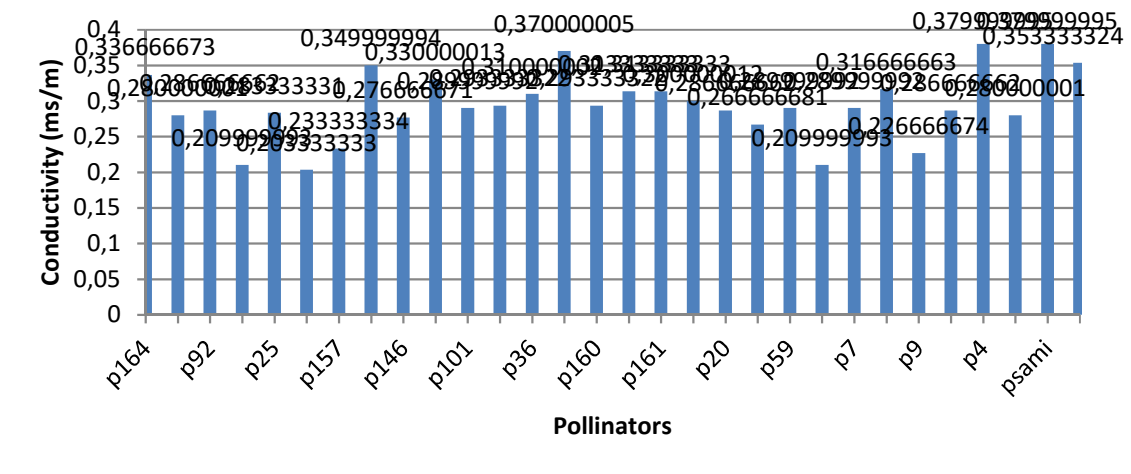

Figure 4. Variation in the value of electrical conductivity of pollen as a function of pollinator

3.5.The water content

The water content of the pollen plays a predominant role in maintaining the viability of the pollen during its conservation. Reducing the water content of a pollen grain greatly reduces its respiration (Rang et al., 2015; Wang et al.,2015; Hoekstra and Bruinsma 1975). The structure of the tricellular pollens gives them a considerable fragility, in particular a low resistance to dehydration; Bicellular pollens are more resistant to severe dehydration (Cerceau-Larrival and Challe 1986). However, Wang et al., (2015) found in larch that severe dehydration causes a significant decline in pollen viability as assessed by conductivity, respiration and germination tests in non-rehydrated pollen. This is partially reversible after rehydration for the conductivity and germination test, but not for the breath test. This could be an indication that there is a limit on the dehydration of the pollen before storage.

From Figure 5, the higher water contents are stored in p39, p146, p9, p92 and p7 (over than 95%). P159 has the lowest percentage of water with 40.8%. It is important to maintain the lowest possible water content throughout the conservation period. The maintenance of low water content of palm pollen during extraction and during storage limits the enzymatic degradation of the endogenous substrates essential for the initial growth of the pollen tube (Stanley and Poostchi, 1961). Storage conditions that maintain the viability limit the action of enzymes that break down simple sugars: in fact, retained palm pollen grains that do not germinate have lower carbohydrate content than those that germinate (Rang et al.,2015).

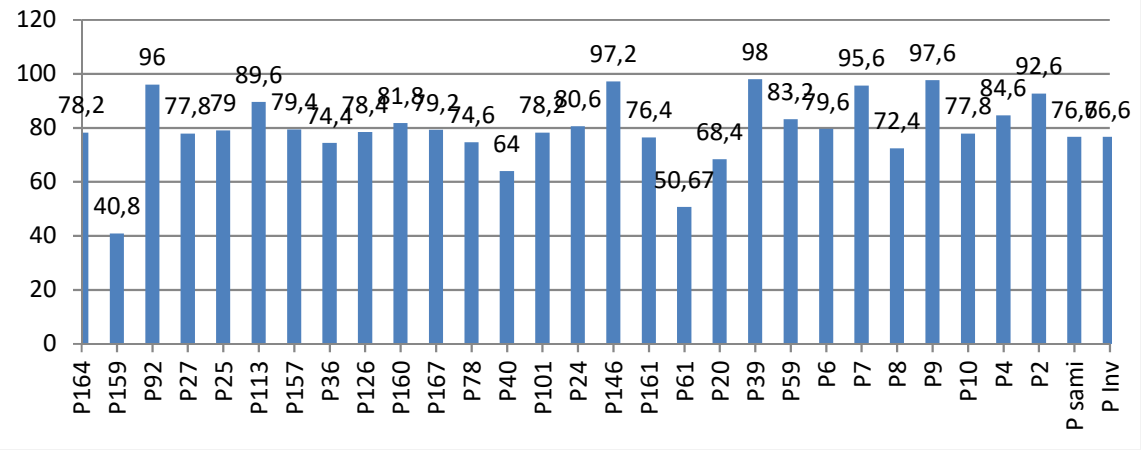

Figure 5. Variation, in value of the moisture content of pollen based on the pollinator

3.6. Effect of conditioning temperature on physiological parameters

3.6.1. Kinetics of pollen germination at room temperature

In order to realize the curve of kinetic rate of germination at ambient temperature, samplings were chosen at the end of the month. The curve illustrated in figure 6 is formed by 10 points. Initial germination rates of fresh pollen were measured from the first sampling for both temperatures (ambient and 4 ° C). The first point of the curve begins towards the end of March and so on for all the other points according to fig. 5 that at the end of the first month the pollinators P Inv and P 2 have kept the rates of germination, the lowest were observed in pollinators P 39 and P 10. Analysis of this figure shows that the rates have decreased between 55 and 60% after 5 months of storage at room temperature. Finally, after 10 months, an average germination rate is recorded which varies between 20 and 10%. The lowest rate of reduction was recorded in pollinator P Inv with a rate of 78%. However, the highest rate was observed in the pollinator P 2 with a rate of 90%. From fig 5 it can be said that by conserving the pollen at ambient temperature a germination rate decreases of up to 90% was recorded relative to the initial levels.

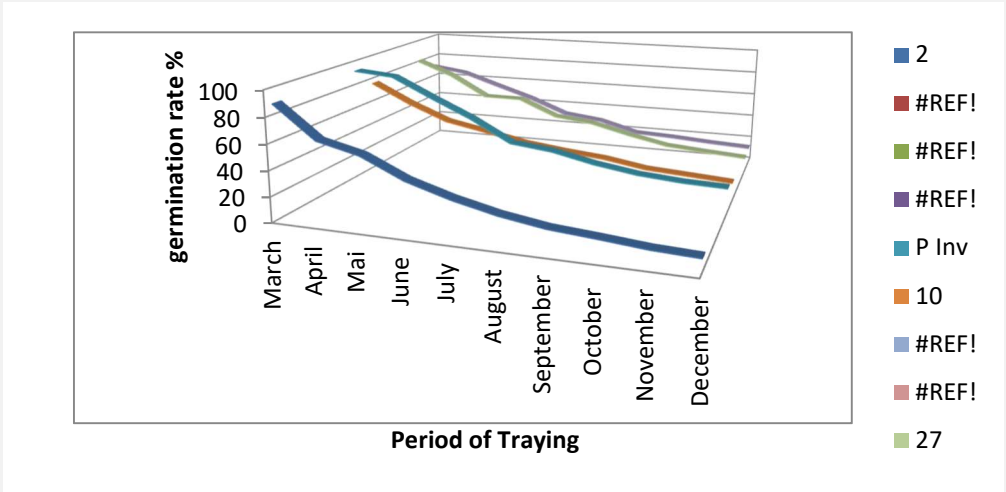

Figure 6. Evaluation of the germination rate of pollen as a function of time at ambient temperature

3.6.2. Kinetics of pollen germination at refrigerated temperature (4°C)

The same protocol and the same individuals were applied for this temperature. From Figure 7, it can be seen that the highest rates were observed in the majority of pollinators, especially P 2 and P 27. The results show that the initial rate is between 94 and 100%, after 5 months of conservation we notice a decrease in half of this rate ranging between 40 and 50%. The highest rate of reduction was observed in the P 27 pollinator with a rate of 52%. Beyond 10 months, a reduction of the germination rate is between 80 and 50% in the majority of the pollinators. From these results, it can be seen that storage at a temperature of 4 ° C recorded an average reduction rate of 30%, while at this rate it was 70% for the ambient temperature.

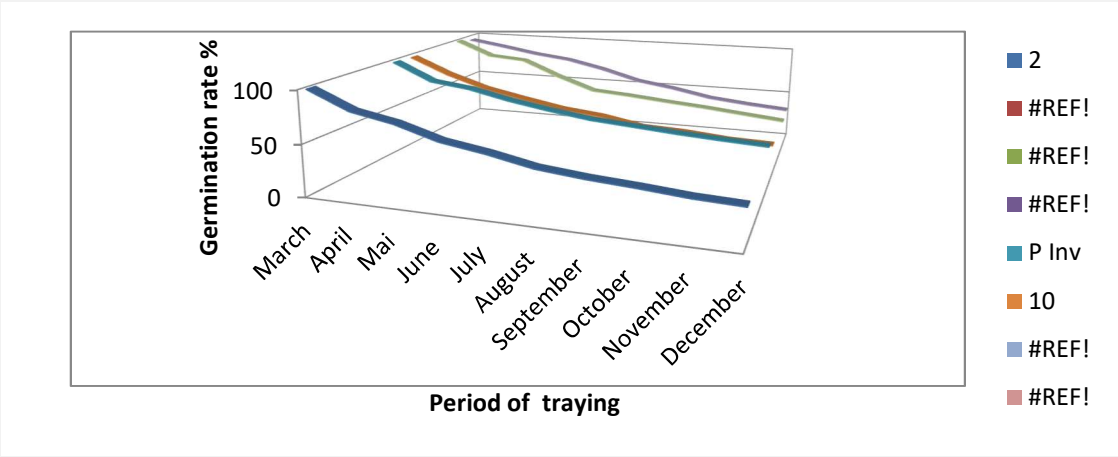

Figure 7. Evaluation of germination rate of pollen as a function of time at temperature of + 4 ° C

3.6.3. Kinetics of pollen viability at room temperature

At the end of the first month, the highest rates were observed in pollinators P2, P193, and P10, the lowest observed in P Inv pollinators. According to Figure 8, it can be seen that the kinetics of the viability rate is characterized by a reduction rate that varies according to the type of pollen. Indeed, after a period of 5 months, the rate of reduction is not very important and varies between 30 to 40%. After 10 months of storage, a significant decrease in the viability rate was observed, reaching up to 82% in the P Inv pollinator, with an average reduction rate varying between 75 and 80%.

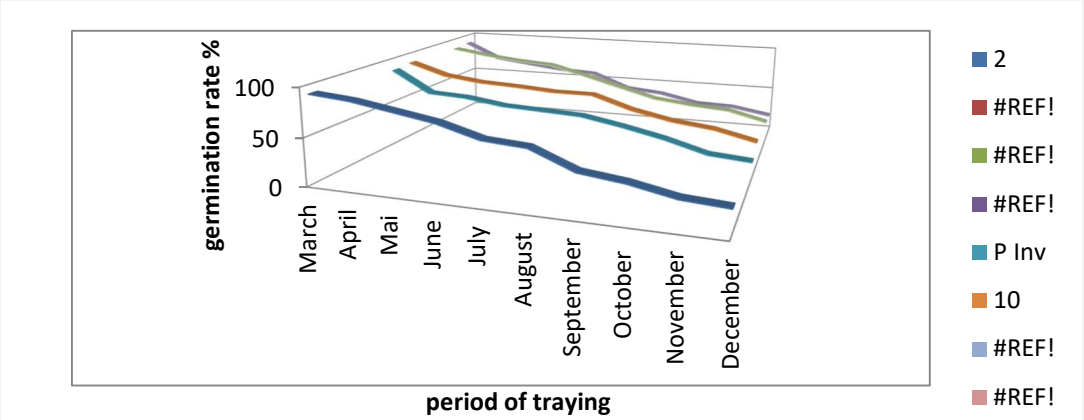

Figure 8. Evaluation of viability rate of pollen as a function of time at ambient temperature

3.6.4. Kinetics of pollen viability at refrigerated temperature (4°C)

The majority of pollinators recorded a high rate of 97-95% initially, with the exception of P Inv pollinators (88%). From Figure 9, it can be seen that, after 5 months, the mean of the viability rate has decreased slightly from the initial state, but it is noted that this decrease does not exceed 20% except poll en P Inv (35%). After a 10-month period It should be noted that the pollen viability rates stored at + 4 ° C did not decrease significantly, the majority of pollinators maintained a viability rate of almost 50% with the exception of P Invi which recorded a reduction rate of 65%. According to these data, it can be concluded that the conservation of pollen at + 4 ° C allows to keep viability levels more or less important. On the contrary, the storage at room temperature has a degrading effect on the viability of the pollen. From the results it can be said that the germination rate data of some pollinators are not compatible with their viability rate. Indeed, P Inv has the highest germination rate at + 4 ° C and its viability rate at + 4°C is the lowest. Thus, there is no positive correlation between the viability rate and the germination rate (Yang et al.,2015).

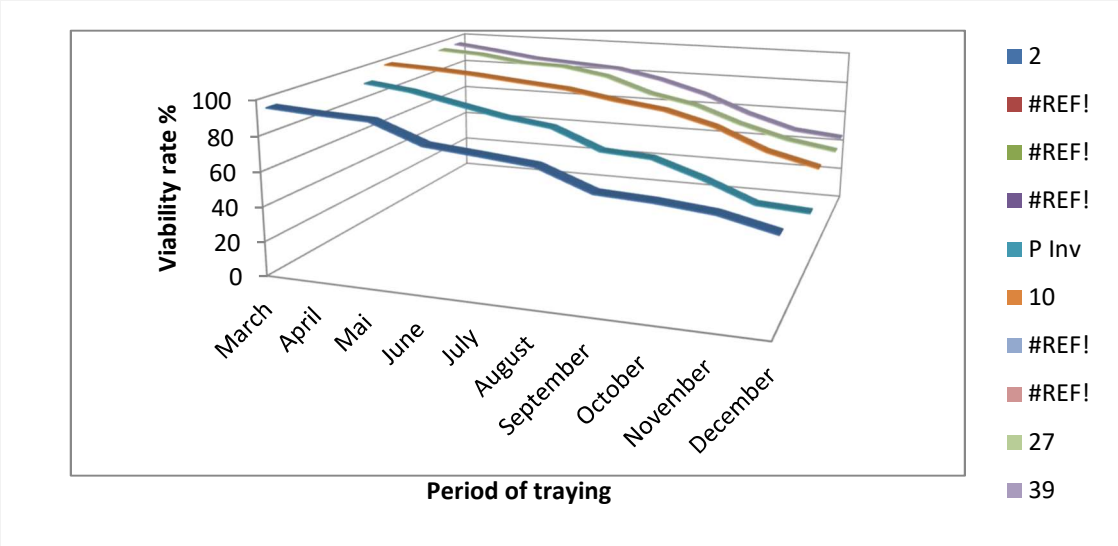

Figure 9: Evaluation of viability rate of pollen as a function of time at temperature of + 4 ° C

Viability or germination tests are used to judge the intrinsic capabilities of the pollen, using staining tests or simple culture media: Monciero medium (1954) and medium Brewbacker and Kwack (1963) modified and used by Furr and Enriquez (1966). Viability tests are used in the United Arab Emirates to test the viability of packaged and marketed pollen (Shabanaet al.1998). The moisture content of fresh pollen in almost all pollinators is between 80 and 95%, Issarakraisila et al (2014). They appear relatively high, which can cause microbial attacks; especially since pollen is rich in proteins and vitamins (Boughediri. 1985). Drying remains a solution to avoid these problems.

A good pollinator should have a pH value close to neutral and the average electrical conductivity (CE), is relatively low. The staining and germination rates appear to be very high. The majority of collected pollens are considered very good because its rates exceed 75%. Yang et al (2015) and Nasr et al (1986) report that good pollinators produce pollens with staining and germination rates above 75%. Our results also confirm the findings of the grower who claims that his pollinators are among the best since a spikelet is enough to pollinate a female inflorescence.

The monitoring of the viability of the pollen, retained by the different methods, allowed us to know the effect of the conservation conditions on pollen viability. Pollen viability rates are

lower than fresh pollen. Pollen kept in the refrigerator keeps its viability better than those of other methods. This result confirms those of Chakrabarti et al (2012) and Gomez et al (2002). For the evolution of the viability of preserved pollen, we can say that they are relatively close, especially at the end of the conservation period. Pollen stored in the ambient temperature rapidly loses its viability compared to those stored in the refrigerator; due to climatic factors and uncontrolled storage conditions (Kwoun et al.,2005). At the end of the summer period, pollen viability decreases for all conservation methods, especially those of pollen stored in the environment.

Pollen germination rates are also lower than fresh pollen. After the summer period, pollen kept in the refrigerator has a high germination rate compared to pollen stored at home or on the farm. Najafi et al (2006) and Nel and Staden (2005) report that the pollen kept in the refrigerator for one year loses between 28 and 69% of its germinative potential. Pollen germination rates decreased significantly at the end of the summer season, especially those retained at home and in operation (Rang et al., 2012; Dafni et al. 2000; Moustapha and El Ajilli 1993). The pollen preserved in the farm quickly loses its germinative potential. We recommend that growers review this method. It should be noted that farmers are used to increasing the number of spikelets per inflorescence in order to compensate for this decrease in germination (Dutta et al. 2013; Abdelgadir et al. 2012).

The study of some physicochemical characteristics of the pollen showed that the fresh pollen is characterized by a neutral pH and a conductivity that does not exceed 0.7 µS / cm. Its humidity is very high, it generally exceeds 75% (Chaudhuryetal.; 2010; Doyl. 2001). Conservation of pollen has an effect on the physicochemical characteristics of the pollen. After the summer period, the pH becomes slightly acidic, whatever the method of conservation. The electrical conductivity increases. The viability and germination rates decrease with all the methods studied except for the conservation of the spikelets in the refrigerator (Rang et al., 2012; Dafni et al. 2000; Broun and Dyer. 1991).

4. Conclusion

Our study is carried out on 30 male date palm pollinators which have undergone a physiological characterization: this characterization revealed a significant difference between the pollinators. Moreover, the tests carried out do not have proportional relations; in fact, the germination test and the viability test are independent. The correlation between the germination test and the conductivity is negative. The comparison between the two methods of pollen conservation allowed us to observe that the temperature + 4 ° C. allows a better conservation of the viability and the germinative rate of pollen of *Phoenix dactilyfera* L.

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