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Optimization of Maintaining Factors of *Senna spectabilis* Pollens: A Bee Species of Adamawa's Flora (Cameroon)

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ABSTRACT

The study's primary goal is to optimize the preservation of germination of *Senna spectabilis* pollens. This large species of bee flora of the Adamawa region is indeed threatened with extinction. The work conducted from November 2006 to May 2007 have focused on testing *in vitro* germination and storage of pollens taken from the study site. The tests required the installation of two solidified media culture: Brewbaker and Kwack (BK) and Heslop-Harrison (HH) media to assess the influence of some physiological factors and storage effects at 10 and -20°C on the germination of pollens. The results obtained show that *Senna spectabilis* pollens germinated preferentially up to 38.36% on BK medium enriched with the optimal concentration of 25% sucrose. The optimal incubation temperature is set at 30°C. The pH 5.6 was more favourable for the germination of pollens with a rate of 32.48%. The pollens showed a high tolerance to desiccation by maintaining after 8 weeks of dehydration a germination level situated at 2.94%. The effects of initial drying on all the 22 weeks of storage were most notable during the periodic tests with improved germination of pollens of one week of drying (13.88%) from the 6th week of storage at +10°C. This improvement has been effective during the first 4 weeks of storage at -20°C. With or without initial drying, pollens stored at 10 and -20°C have germinated at length during the 22 weeks of storage in general.

Key words: Pollen, germination, desiccation, storage

INTRODUCTION

The Adamawa region is part of the Sudano Guinean zone of Africa which generally contains a spectacular diversity of plants and floral phenotypes (Kay and Schemske, 2003). This region of the northern part of Cameroon produced approximately 2 to 4.5 million liters of honey per year through a network estimated in 2005 to about 10,000 beekeepers mainly distributed in rural areas (SNV, 2006). It's dense and diversified flora (Letouzey, 1979), enjoys a favourable climate for beekeeping. However, the combination of effects related to global warming associated with anthropogenic recurring actions: uncontrolled slaughter of species, wildfires and grazing activities lowered the prosperity of beekeeping in the region. It is known that the influence of human activities on vegetation dynamics and the ecosystem evolves with population growth and constitutes a threat for the survival of several useful species (Lykke *et al*., 1999; Backer *et al*., 2004; Tchotsoua, 2005). Indeed, the natural factors affecting environment and human actions can alter beekeeping

ability of the region by their intervention plans and land use or farming practices (SNV, 2006). Thus, important nectar plants such as the genera species *Senna*, *Callistemon*, *Vitellaria*, *Hymenocardia*, etc, are now threatened in several localities of the region in this part of Cameroon.

To this end, biotechnological methods of preservation of *in vitro* germination capacity of pollens on significant periods may extend over months or years to create and maintain ambitious regeneration schemes and genetic improvement of threatened plant species. It was possible to get through these processes *in vitro* germination of frozen pollens of *Dacryodes edulis* after two years of storage (Youmbi *et al*., 1998). In *Petunia hybrida* (Farcy *et al*., 1990) and in *Radiata* pine (Siregar and Sweet, 2000), germination extended to one year of storage. A good conservation in the best standards should allow the return of characters who have guided the choice of the collector during a controlled pollination (Cerceau-Larrival, 1990). In other words, these characters must persist over time (Youmbi, 1993). To achieve this, the storage of pollens at low temperatures requires that those which are tolerant to dehydration are previously dehydrated (Taylor and Hepler, 1997; Engelmann and Dussert, 2000). Thus made, the storage will facilitate the transport and distribution of pollens, allowing hybridization between plants scattered in space and shifted in flowering time (Charrier, 1990). The use of constituted pollens bank will allow species bearing few amount of these male gametophytes to accumulate a significant quantity in order to contribute to delayed pollination for a considerable number of plants (Youmbi, 1993). This work is aimed to optimize the preservation of pollens germination capacity of *Senna spectabilis*. The specific objectives will help achieve the optimum parameters of germination and storage that are: culture medium, concentration of sucrose, culture temperature, pH of culture and storage temperature.

MATERIALS AND METHODS

Study site: The Adamawa is a highlands region of the northern part of Cameroon (LN 6°02'-7°38' and LE 11°36'-14°57'). It covers an area of 63,691 km². The altitude is situated between 1000 and 2000 m and gives a relatively cool climate with a varied temperature between 22 and 25°C. This region belongs to the sudano guinean zone of Africa.

Savannah is the main vegetation. It presents a better afforestation in the southern part which gradually deteriorates towards the north. *Senna spectabilis* species blooms regularly from November to April.

Plant material: The plant material consists of *Senna spectabilis* pollens. The removal of pollens on the site is been done by the anthers collection which is done early in the morning (7 o'clock) before their dehiscence. They are kept in special bags (capable of maintaining pollens viability) and once in the laboratory, the pollens from the anthers are extracted using forceps and spatulas.

Germination tests *in vitro*: The two media used were those of Brewbaker and Kwack (1963) and Heslop-Harrison (1979). A volume of 12.5 mL of each stock solution is removed and heated to which agar is added to a concentration of 1% (0.125 g). After cooling, sucrose (SOSUCAM) is added in varying concentrations (0, 5, 10, 15, 20, 25, 30, 35%). Regarding the determination of favourable pH for the germination of pollens, the BK medium prepared as indicated above is adjusted to different pH with sodium hydroxide (NaOH 0.1 N) and hydrochloric acid (HCl 0,1N). Reading the pH of the solution was made using a pH meter of HANNA mark. Different values of incubation temperatures (20, 25, 30, 35 and 40°C) were tested to identify the one that allows for optimal germination of pollens. The pollens contained in open pillboxes were introduced into a desiccator

containing blue silica crystals during variable lengths (0, 1, 2, 3 weeks) for dehydration. The conservation of dehydrated pollens and non-dehydrated controls was made simultaneously at -20°C (freezer of Aston mark) and +10°C (refrigerator of Aston mark). The germination tests *in vitro* were performed each week. The different prepared and readjusted media are flown on slides and left to cool for several minutes before being sown with pollens and cultured for 24 h. After this duration, the slides are brought out of the incubator and stained with Alexander (1969), then they are coated with plates and then observed under the light microscope of Olympus mark to determine the pollens germination rate.

The germinated pollens are counted under the light microscope and the percentage is measured according to a total number of pollens (germinated or not) counted on each slide. This number is greater than or equal to 400. A pollen grain is considered germinated when the pollen tube length is more than half its diameter (Visser, 1955; Bocquel, 1995).

Data analysis: The STATGRAPHICS Plus 5.0 statistical software allowed for the analysis of variances of the obtained data. The multiple comparison tests were performed using Duncan's test.

RESULTS

Influence of culture medium and the sucrose concentration on the expression of pollens germination capacity: On the HH culture medium, the germination percentage of pollen grows relatively high to the concentration of control (no sugar) at the concentration of 15%, moving thus from 0.81±0.07 to 19.69±2.13% (Fig. 1). Thereafter, the pollens germination capacity stabilizes slightly at the rates of 15 and 20% where the germinations are highest with 19.69±2.13 and 18.49±0.88% before declining significantly to the value of 2.85±0.15% at the maximum sugar concentration (35%). The pollens germination capacity on BK medium produced expression levels similar to those obtained on the HH medium from control sugar concentration to 20% (Fig. 1). Indeed, the difference in the level of expression of the pollens germination on both culture media in this interval is less than 1.7%. Beyond a sucrose concentration of 20%, the BK culture medium is characterized by a sharp rise in the percentage of pollens germination. In the second interval of sucrose concentration (25-35%) compared to the first (0-20%), the expression of pollens germination capacity on BK medium is significantly higher than that of pollens cultured on the HH medium

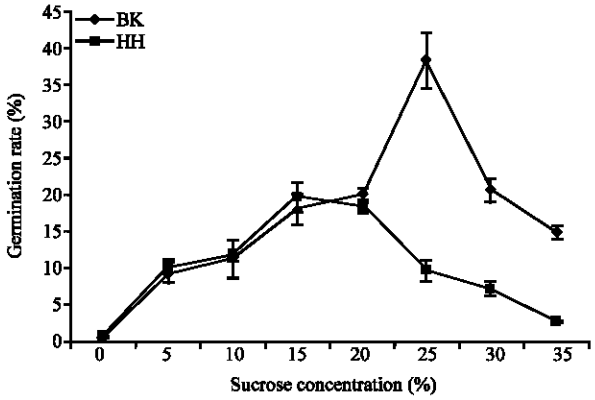

Fig. 1: Evaluation of the influence of culture medium and sucrose concentration on the expression of pollens germination capacity

Table 1: Influence of incubation temperature on the expression of pollens germination capacity

Species	20°C	25°C	30°C	35°C	40°C
S. spectabilis	27.50±3.44b	36.85±5.09c	38.36±3.7c	15.61±2.01a	09.88±0.81a

Duncan's test (0.05): the numbers affected with the same letter are not significantly different

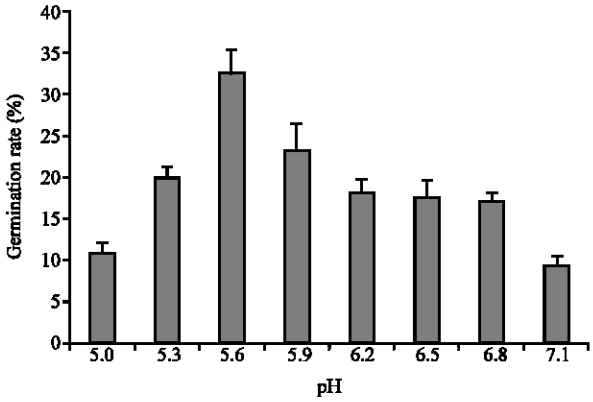

Fig. 2: Influence of pH on the expression of pollens germination capacity

(Fig. 1). To this effect, the smallest difference between germination percentages on both media between 25 and 35% sucrose is 12%, the highest being estimated at more than 28% at 25% of sucrose. This concentration of sucrose which yielded 38.36±3.79% of pollens germination on BK medium is the optimal value of sugar for efficient expression of germination on this medium.

Influence of culture temperature on the expression of pollens germination capacity: Temperatures of 25 and 30°C allowed to have the best germination percentages respectively 36.85±5.09 and 38.36±3.7% (Table 1). There is however a rather strong decrease of pollens germination at high temperatures of 35 and 40°C. Compared to the percentage of germination obtained at the optimum temperature of 30°C, there is indeed a decline in germination percentage of about 60-74% at temperatures of 35°C with respectively the germination rates of 15.61±2.01% at 40°C and 9.88±0.81% at 45°C. The culture temperature generally evolves with increase in the percentage of pollens germination until the optimal value of 30°C from which the values of higher temperatures (35 and 40°C) tend to produce inhibitors effects on the germination of pollens (Table 1).

Effect of pH on the expression of pollens germination capacity: The test of pH influence on the pollens germination capacity results firstly by a rapid growth in the germination percentage between pH 5 and pH 5.6 (Fig. 2). The germination percentage was in fact tripled between these pH values, moving respectively from 10.83±1.17% at pH 5 to 32.48±2.76% at pH 5.6. The second phase which extends from pH 5.6 to 7.1 is characterized by a general decrease in varied magnitude. Thus, the decline occurs first significantly between pH 5.6 and 5.9 of the order of 29%, respectively from 32.48±2.76 to 23.11±3.3% (Fig. 2). It then stabilizes around 18% of germination at pH 6.2 to 6.8 before becoming more sensitive between pH 6.8 and 7.1 of the order of 45% where the germination percentage decreases from 17.06±0.96 to 9.37±1.03%. Of the 8 values of pH tested, pH 5.6 better stimulated the expression of pollens germination capacity (32.48±2.76%).

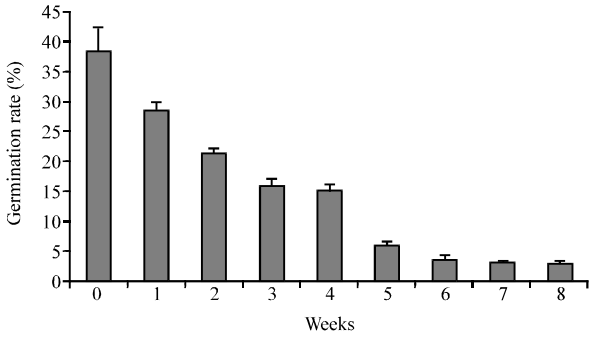

Fig. 3: Evaluating effects of desiccation time over the expression of pollens germination capacity

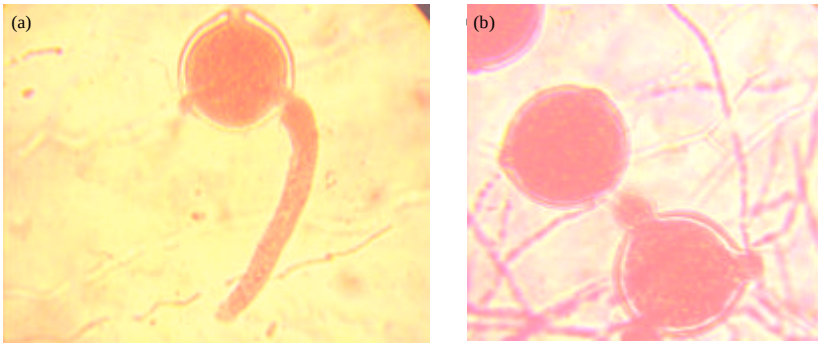

Fig. 4: (a) Germinated pollen after 1 week of drying and (b) dominant aspect of pollen after 8 weeks of drying

Effect of drying time on the germination of pollens: The influence of the drying time on the germination of pollens results in a gradual decline in their ability to germinate (Fig. 3). From one week to another during the 8 weeks of drying, the declines were generally quite sensitive to the order of at least 15%, except those held between the 3rd and 4th week with 3% (15, 7±1.48% against 15.17±1.02%), between the 6th and 7th with 11% (3.58±0.7% against 3.2±0.28%) and between the 7th and 8th with 8% (3.2±0.28% against 2.94±0.41%). As for comparing the level of expression of dehydrated pollens germination over time compared to the control (without dehydration), we note that dehydration caused a larger decrease proportional to the drying time. Indeed, if after 1 and 2 weeks of drying, pollens lose respectively 25 and 44% of germination capacity (38.36±3.79% against 28.6±1.27% and 38.36±3.79% against 21.45±0.71%), this loss amounts to more than 92% after 7 and 8 weeks (38.36±3.79% against 3.2±0.28% and 38.36±3.79% against 2.94±0.41%) (Fig. 3, 4a, b). However, it is important to note that no complete inhibition of pollens germination is before or after the 8th week of drying during the experiment.

Effect of initial drying on the storage of pollens at +10 and -20°C: The storage of pollens initially dried in the refrigerator (10°C) after 2 and 4 weeks did not permit an increase in their germination capacity. Indeed, the germination of pollens stored without initial drying (control) remained higher than those of the pollens of 3 drying times during periods of storage (22.47±2.9%>21.51±1 57% and 20.79±1.46%>18.62±1.66%) (Fig. 5a). By cons, during the following four weeks, germination percentages of the series of three batches of dehydrated pollens were

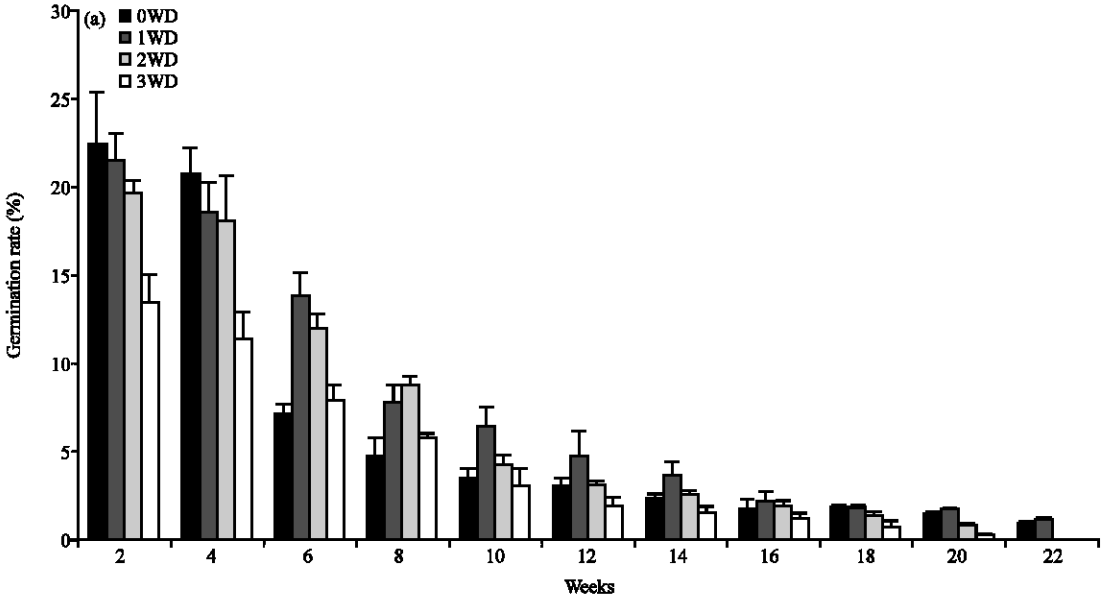

Fig. 5: (a) Influence of initial drying on the pollens storage at +10°C and (b) evaluation of critical storage period (at 10°C) of initially dehydrated pollens

higher than those of witness who have indeed experienced a sharp decline of 66% (20.79±1.46% against 7.14±0.5%) at the end of the 6th week. If the superiority of the pollens germination of one and two weeks of drying continued until 10 weeks of storage compared with controls, we note by cons that beyond that time and until week 22, only one week of pollens drying maintains a relatively higher germination than controls (Fig. 5a). It is also observed a complete inhibition of pollens germination of one and two weeks of drying after 22 weeks of storage at 10°C. Thus, this storage period remains their critical period of germination (Fig. 5a, b). Given that pollens witnesses and those of a week of drying germinated at length during the time tested (22 weeks), we noticed that critical periods of storage of these two types of pollen are over 22 weeks (Fig. 5a and b). For storage in the freezer at -20°C, comparing the behaviour of pollens germination between witnesses and the pollens of a week of drying leads to a remark relatively opposite to that made during cooling to 10°C . During storage at -20°C, the germination rate of pollens from a week of drying were relatively higher during the first 6 weeks with those of control (no initial drying) (Fig. 6a). However, beyond that time, the storage of different batches of pollens results in an overall gradual decrease and mainly by the relative superiority of witnesses pollens germination on the pollens of one week drying. This superiority is more sensitive on the pollens germination capacity of 2 and 3 weeks drying (Fig. 6a). In general, the initial drying before storing pollens at -20°C will produce a truly remarkable and positive influence with the pollens of one week drying during the first 3 phases of testing (6 weeks). The particularity of storage at -20°C is found throughout the

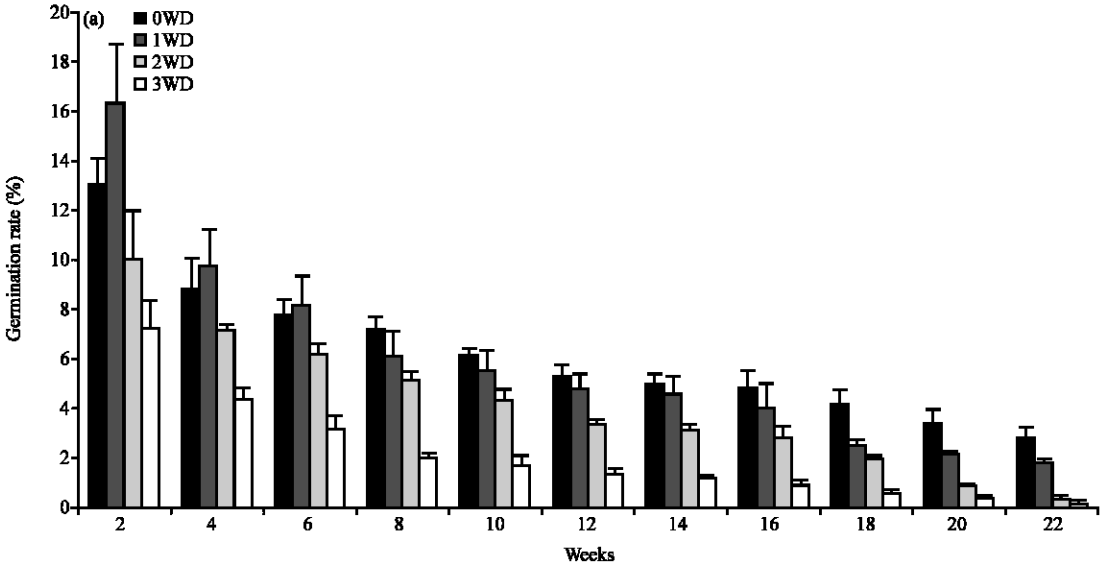

Fig. 6: (a) Influence of initial drying on the pollens storage at -20°C and (b) evaluation of storage critical period (at -20°C) of initially dehydrated pollens

sustainable germination of all four batches of pollens on the entire test period (22 weeks). This suggests that different critical periods of storage are all located beyond 22 weeks (Fig. 6a, b).

DISCUSSION

The BK medium culture has permitted to obtain the highest rates of pollens germination of *Senna spectabilis* in general. If this observation corroborates that of Youmbi *et al.* (1998) during a similar study in *Dacryodes edulis*, it stands out against the results of Cerceau-Larrival and Challe (1986) in *Dactylis glomerata* and Youmbi (2005) in three varieties of *Zea mays*, where the HH medium was more favourable to the germination of pollens. The *Dactylis glomerata* and *Zea mays* species belonging to gramineae family, the strong ability of expressing their pollen germination on HH medium may seem understandable in that this medium was originally developed by Heslop-Harrison (1979) for the germination of pollens of *Secale cereale*, which is also a species of gramineae family. The pollens of this species may indeed have physiological abilities close to those of *Dactylis glomerata* and *Zea mays*, but different from those of *Senna spectabilis* which is a Caesalpiniaceae. Thus, the differences that occur in the responses of various species in the culture media may have several causes more or less varied. For a given medium, these differences may reside in its mineral composition or in its composition of organic substances. In the same medium, they can depend on such environmental stresses (drought, soil acidity, mineral content of soil, etc.) affecting the plant that bears the pollen. Indeed, as noted by Charrier (1990), a good preservation of pollen depends on the genetic structure of the plant and its mode of reproduction. The sucrose

concentration, nutrition source for pollen tube (Visser, 1955; O'kelley, 1955; Baloch *et al*., 2001) is a key factor that affects significantly the level of expression of germination capacity. The sugar optimum rate of 25% obtained in this work is identical to that obtained for the pollens germination of cotton (Choudhry and Akhmedova, 1982) and *Okra* species (Philomena and David, 1984). It differs from the rate achieved during the work of Bocquel (1995) in *Betula verrucosa* (10%), of Baloch *et al*. (2001) in a variety of cotton (40%) of Youmbi *et al*. (2005) in Varieties of *Zea mays*: Exp_1 5057 (10%), Exp_1 24 and Tuxpeño Sequia (15%). In germinating up to 38.36% in the optimal nutrient conditions, the pollens of *Senna spectabilis* can be range in the series of pollens with an average capacity of germination. Indeed, if the optimal rate of germination (38.36%) remains lower than those obtained by Kendall (1967) in *Trifolium pratense* (90%), Walden (1994) and Torres *et al*. (1995) in *Zea mays* (90%), Bocquel (1995) in *Betula verrucosa* (85%), Youmbi *et al*. (1998) in *Dacryodes edulis* (96%), it is nevertheless higher than those obtained on the same culture medium (BK) by Youmbi *et al*., (2005) in three cultivars of *Zea mays* (30.55, 25.27, 19.54%). The culture temperature of 30°C required to obtain the optimum germination rate has remained consistent with that required in similar works by Youmbi (1993) in three tropical species: *Catharanthus roseus*, *Adenium obesum* and *Pachipodium lamerei*, by Tamnet (2002) in two diploid varieties of *Musa acuminata* and Stone *et al*. (2004) among Conospermum species. It was also noted during this work that the pollens of *Senna spectabilis* germinate preferentially in weak acid medium at pH 5.6. This pH value is lower than those reported by Youmbi (1993) in *Pachipodium lamerei* (6.0) and *Adenium obesum* (6.2), Fan *et al*. (2001) in *Arabidopsis thaliana* (5.8) and Tamnet (2002) in the Troncata variety (5.9) of *Musa acuminata*. The pH values of these authors show that pollens of their species prefer media that are more less acidic. The persistence of pollens germination of *Senna spectabilis* in spite of its gradual decline and significant spread over eight weeks of drying have shown that pollens got relatively sustainable viability. This course is significantly different from observations made in some cultivars of *Zea mays* during Tedjacno (2002) and Youmbi *et al*. (2005) works. Indeed, these authors had observed the deletion of viability and therefore necessarily the inhibition of pollens germination capacity after only five hours of drying. In *Senna spectabilis*, this result suggests that the pollens of this species would have relatively low moisture content at anthesis (less than 40%). It is known for example that the normal amount of moisture in pollens at anthesis must be less than 40% and pollens that have requirements beyond this rate are very sensitive to water stress (Digonnet-Kerhoas and Gay, 1990). The major consequence of this low moisture content in pollens of *Senna spectabilis* could result in their long viability and persistence of their germination capacity during storage in refrigerator (10°C) and freezer (-20°C). It has been shown that pollen viability is linked to intracellular ice formation (Ichikawa and Shides, 1971) and that intracellular ice during the freezing might cause damage affecting the exit of the pollen tube (Mazur, 1984; Charrier, 1990). Given the low natural humidity that *Senna spectabilis* pollens could have, it was noticed during this experiment that the pollens which has not undergone initial drying (control) were not completely inhibited during the 22 weeks of test at both temperature of storage (10°C and -20°C). The crystallization of intracellular water was indeed less important compared to what has been observed in cultivars Exp_1 and Tuxpeño Sequia in *Zea mays* where an initial dehydration at 5 to 10 h is required to extend the viability of pollens stored at 10°C to 20 days (Youmbi *et al*., 2005). Thus, the initial drying before storing pollens, usually aimed at reducing their cytoplasmic content in water to prevent crystallization and thereby increase their longevity has not produced particularly noticeable effects on the entire duration of storage. However, the period of 1 week of

drying significantly improved the germination of pollens during the periodic tests from the end of the sixth week of storage at 10°C. This fact has only been observed during the first 4 weeks of storage at -20°C. It seems important in the earlier works in this species to significantly extend the observations beyond 22 weeks to better understand the effects of initial drying on pollens stored at 10 and -20°C.

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