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Study of Some Factors of Conservation of Pollens of Two Plant Species (*Callistemon rigidus* and *Hymenocardia acida*) of Bee Flora of Adamawa (Cameroon)

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Abstract: To contribute to the preservation of the bee flora of Adamawa, a study to determine the optimal conditions for preservation of *Callistemon rigidus* and *Hymenocardia acida* pollens, two endangered bee species, was conducted from March 2006 to March 2007 in this region. The study began by anthers collection at the experimental site and they were brought to the laboratory where fresh pollens are collected and undergo *in vitro* germination and storage tests. These tests have required the installation of two solidified media culture: Brewbaker and Kwack (BK) and Heslop-Harrison (HH) media to evaluate pollens germination under the influence of some physiological factors and assess the influence of storage at +10 and -20°C with and without initial drying. The results show that sucrose concentrations of 10 and 15% on BK medium has produced respectively 60.69±3.1 and 04.49±0.21% as the highest values of germination percentage in *Callistemon rigidus* and *Hymenocardia acida*. Temperatures of 30 and 25°C which produced 60.69±2.53 and 04.25±0.29% of germination and pH 5 with 55.14±4.14% and 6.8 with 04.54±0.6% were respectively favourable in the same order for the germination of both species of pollens. Time for a week of drying allowed the extension of storage time of both species of pollens as from +10 to -20°C. The *Callistemon rigidus* pollens were generally more tolerant to the storage at-20°C showing the critical period of storage exceeding 22 weeks while those of *Hymenocardia acida* were less tolerant to both temperatures with most critical period of storage established to 8 weeks.

Key words: Pollen, germination, drying time, storage, bee plants

INTRODUCTION

The largest quantity of honey and other bee products consumed in Cameroon and neighbouring countries come from the Adamawa region (Tchuengem, 2004; SNV, 2006). In 2005, it was evaluated 10000 the number of beekeepers in this region producing a quantity of honey estimated from 2 to 4.5 million L year^{-1} (SNV, 2006). This production could be significantly increased due to the diversity of its flora (Letouzey, 1979) and its climate which is particularly favourable to the production of bees (Tchuengem, 2004). Indeed, the tropical regions of which the sudano-guinean zone of Africa carry a spectacular diversity of plant species and floral phenotypes (Kay and Schemske, 2003). However, extensive studies of the dynamics of the natural environment in Adamawa (Tchotsoua, 2005) show up significant degradations suffered plant layer whose greatest concern are bushfires, uncontrolled slaughter of species and grazing activities. Similar threats identified during a study of the floristic diversity of the East Central region of Burkina Faso by Ky *et al*. (2009) show more common difficulties encountered by the flora balance of Sahelian regions of Africa in general. In Adamawa region, *Callistemon rigidus* (Myrtaceae) and *Hymenocardia acida* (Anacardiaceae) species are widely sought plants by honey bees (*Apis mellifera*) (Tchuenguem *et al*., 1997; Djonwangwe, 2002). Well protected and deprived from the threats mentioned above, they can contribute more to preservation of beekeeping activities and to quantitative and qualitative improvement of honey production and derived products (propolis and wax in particular). Biotechnological methods of preservation of pollen germination on significant periods exceeding several months or years constitute the basis of numerous regeneration schemes and breeding of endangered species. The preservation of conditions of tube germination *in vitro* of preserved pollens in endangered species could facilitate their transportation and distribution as well as help to make crosses between species that are distant in space and been shifted in flowering time (Cerceau-Larrival, 1990; Charrier, 1990). Storage also allows for plants with anthers bearing very few pollens to accumulate a quantity that may contribute to pollination delayed for a considerable number of plants

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(Youmbi, 1993). To contribute effectively to the search for solution that can enable the preservation and extension of *Callistemon rigidus* and *Hymenocardia acida* species, the main objective of this work is to determine the optimal storage of pollens of these two species of bee flora in the Adamawa. The specific objectives will be to determine the optimal parameters of germination and storage of pollens (culture medium, sucrose concentration, incubation temperature, pH of culture medium and storage medium).

MATERIALS AND METHODS

Study site: The Adamawa is a region of highlands of the Northern part of Cameroon (LN 6° 02'-7° 38' and LE 11° 36'-14° 57') (Fig. 1). It covers an area of 63,691 km². The altitude is situated between 1000 and 2000 m and gives a relatively cool climate where the temperature varies 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.

Callistemon rigidus flowers regularly throughout the year while *Hymenocardia acida* blooms from November to February 2007.

Plant material: The plant material consists of *Callistemon rigidus* and *Hymenocardia acida* pollens. These are bee plants species abundant on the study site. The removal of pollens on the site is been done by the anthers collection which is effected early in the morning (7 o'clock) before their dehiscence. They are kept in special bags (capable of maintaining the viability of pollens) 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 brought hot 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 and 35%). Regarding the determination of pH favourable 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 dessicator 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 media prepared and readjusted are flowed on slides and left to cool for several minutes before being

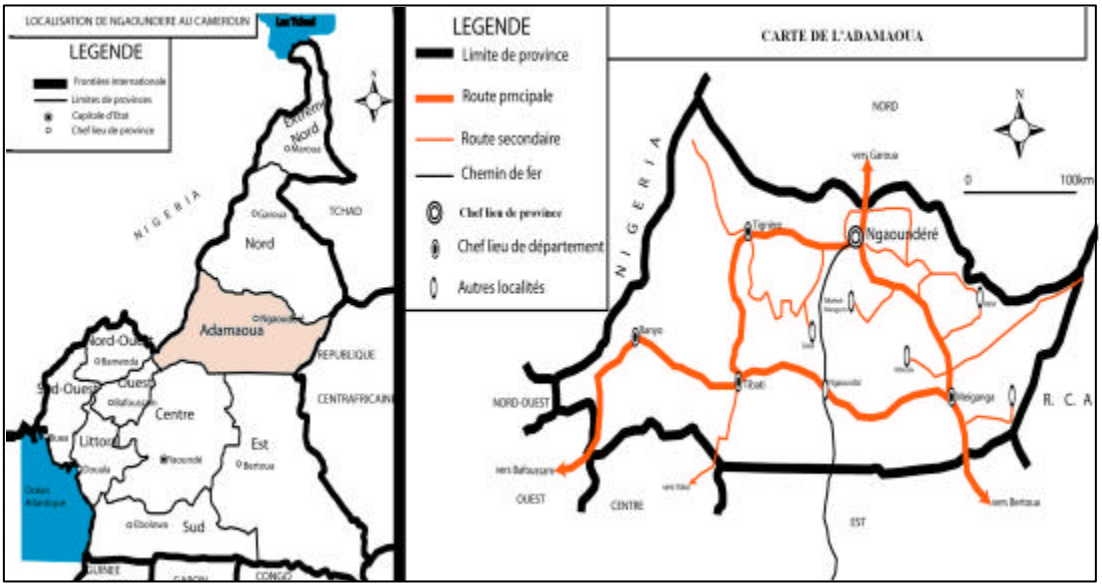

Fig. 1: Map of Adamawa region in Cameroon

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 germination rate of pollens.

The germinated pollens are counted under the light microscope and the percentage is measured reference 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 SPSS 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 sucrose concentration on pollens germination: In *Callistemon rigidus* species, the germination rate of cultured pollens on the BK medium increases with rising rates of sucrose up to the value of 10% with 60.69±3.1% of germination (Fig. 2). The sucrose concentrations which are greater than 10% have resulted to a gradual decline in the germination of pollens. On the HH culture medium, the maximum value of germination of pollens (36.65±2.11%) is obtained with 5% sucrose. HH medium is more than five times less favourable for the germination of pollens than the BK medium (3.4±0.66% against 18.11±1.41%). However, the minimum rate of 5% sucrose can reverse this (Fig. 2). Indeed, this rate is more favourable for the germination of pollens in the HH medium than in BK (36.65±2.11% against 29.39±2.08%). A final reversal of the performance is observed in the expression of pollens germination capacity on both media at 10% sucrose. There is an appreciable superiority in the germination rate of pollens on BK medium from this sucrose concentration. In *Hymenocardia acida* species, we notice that the percentage of germination in BK medium grows until the germination value of 04.49±0.21% at 15% sucrose (Fig. 3). This germination percentage is undergoing a rapid decline in concentrations higher than 20 and 25% sucrose (01.92±0.15 and 0.48±0.08%, respectively) and becomes zero at 30% sucrose. The curve of results obtained on this medium is a bell curve just like on the HH medium. It is noticed on the HH medium that the germination percentage increases moderately from the control value in sugar (0%) to the optimal rate of 15% with 03.06±0.16% of germination

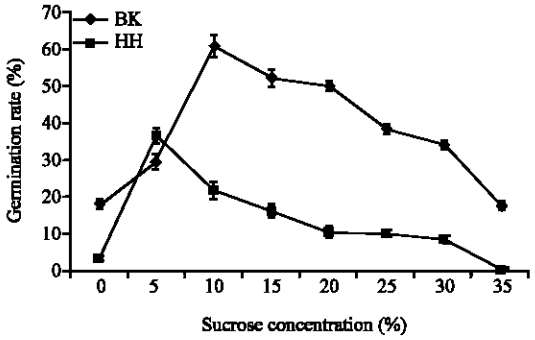

Fig. 2: Influence of base medium and sucrose concentrations on the germination of *Callistemon rigidus* pollens

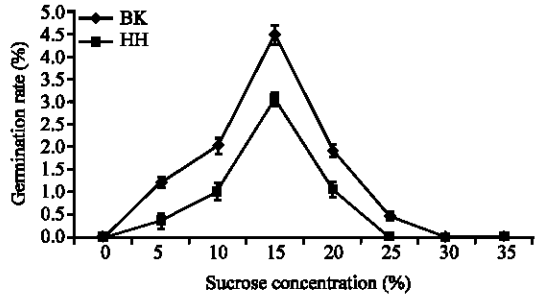

Fig. 3: Influence of base medium and sucrose concentrations on the germination of *Hymenocardia acida* pollens

before declining to 01.07±0.18% at 20% sucrose and vanishes at 25% (Fig. 3). Generally, the pollens germination of both species on BK medium is higher than that obtained in HH medium (Fig. 2, 3). The concentration of 15% sucrose proved the most optimal for germination on both media.

Influence of culture temperature on the germination capacity of pollens: The culture's temperature of 30°C allows for a better expression (60.69±2.53%) of germination capacity of *Callistemon rigidus* pollens (Table 1). The high temperatures of 35 and 40°C are marked by a sharp decrease respectively of 80 and 84% (60.69±2.53% to 12.42±1.13% and 60.69±2.53% to 09.75±1.46%) of percentage of optimal germination of pollens of this species obtained at 30°C. In *Hymenocardia acida*, the temperature of 25°C is the most optimal for the germination of pollens (4.25±0.29%) (Table 1). The complete inhibition of germination in this species occurs at the temperature of 40°C. In the pollens of both species, it is noticed that the temperature zone ranges between 25 and 30°C is more favourable for their germination

Table 1: Pollen germination according to incubation temperature

	Incubation temperature (°C)				
Species	20	25	30	35	40
C. rigidus	16.66±2.37^{a,a}	22.40±2.39^{b,a}	60.69±2.53^{c,a}	12.42±1.13^{d,a}	09.75±1.46^{d,a}
H. acida	01.74±0.11^{a,b}	04.25±0.29^{b,b}	02.75±0.13^{c,b}	0.89±0.09^{a,b}	00±00^{a,b}

•Duncan Test (0.05): The numbers affected with the same letter to the left of the decimal point are not significantly different in the row and in the column if they are affected with the same letter to the right of the decimal point

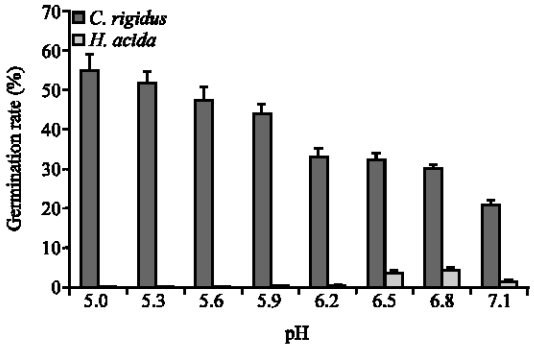

Fig. 4: pH influence on the germination of *Callistemon rigidus* and *Hymenocardia acida* pollens

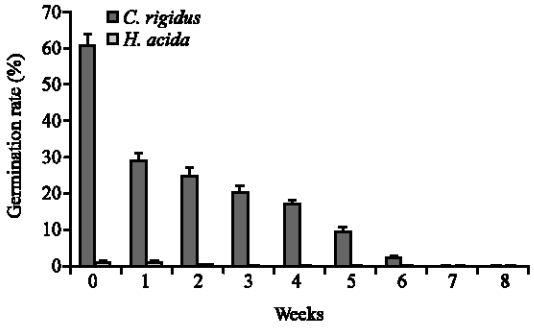

Fig. 5: Effect on drying time on the germination of *Callistemon rigidus* and *Hymenocardia acida* pollens

despite the low germination capacity of *Hymenocardia acida* pollens.

Effect of pH on the germination of pollens: The *Callistemon rigidus* pollens seeded onto the culture media at pH 5 and 5.3 germinate more than 50% (55.14±4.14 and 51.76±2.93% respectively) (Fig. 4). Even if germination decreases and falls below the rate of 50% at the higher values of pH (from 5.6 to pH 7.1), there is generally a low amplitude decrease (<11%) between the two neighbouring values of pH. As for the *Hymenocardia acida* pollens, the maxima germination values were recorded at pH 6.5 and 6.8 with respective rates of 3.58±0.59 and 04.54±0.6% (Fig. 4). The previous values of pH (pH 5 to 6.2) have inhibited the pollens germination of this species. The pH test permits to observe that the pollens of both species germinate in two areas of markedly different pH. The pollens of *Callistemon rigidus* show their preference for the culture medium with acidic pH (5) while those of *Hymenocardia acida* germinate at near neutral pH (6.8).

Influence of drying on the germination capacity of pollens: After the first 7 days of drying, the loss of water that followed caused the sudden fall of about 52% (60.69±3.1 to 29.03±1.93%) on the germination of pollens of *Callistemon rigidus* (Fig. 5). This decrease is continuous and less strong (<20%) over the next 3 weeks. The 5th and 6th week of drying are characterized by significant decreases of 44 and 75% respectively of the germination capacity of pollens of this species (17.1±1.18% to 09.62±1.08% and 0.962±1.08% to 0.236±0.43%). This germination capacity is completely inhibited after 7 weeks of desiccation. Inhibition is more precocious in the pollens of *Hymenocardia acida* after only 3 weeks of drying at which 84% of germination capacities of pollen controls is lost at the end of the first week (Fig. 5). The pollens of both species at different periods of drying have generally shown a main similarity marked by a great decrease in their ability to germinate (>50%) after a week of drying.

Effect of drying on initial pollens storage at +10°C and -20°C: In *Callistemon rigidus*, storage of dehydrated pollen to +10° C for one and two weeks enhances their germination rate compared to pollen witnesses which have not undergone initial drying and are stored at the same temperature (Fig. 6). The effects of 3 weeks of drying on the storage of pollen at 10°C are less remarkable. The desiccation of *Callistemon rigidus* pollens involves the extension of the maximal time storage at 10°C for 6 weeks for the pollens of 1 and 2 weeks of drying and 4 weeks for the pollens of three weeks of drying. Indeed, after 8 weeks of storage at 10°C, pollens germination capacity is inhibited but the pollens of one and two weeks of drying continues to germinate until 12 weeks and are inhibited after 14 weeks storage (Fig. 6). In *Hymenocardia acida*, the effects of initial drying on the storage of pollens at 10°C are relatively similar to those observed in *Callistemon rigidus* despite the very low germination of pollens (Fig. 7). In this species, the effects of drying during a week are more remarkable than any other period

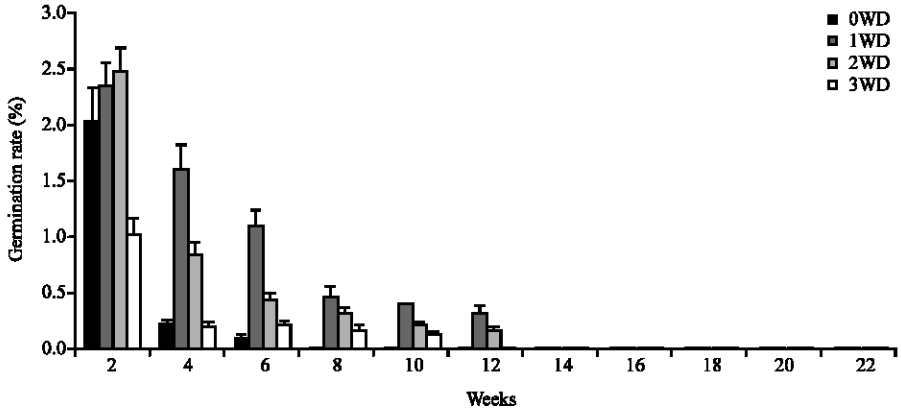

Fig. 6: Influence of drying time (period) on the germinating power of cooled (+10°C) *Callistemon rigidus* pollens

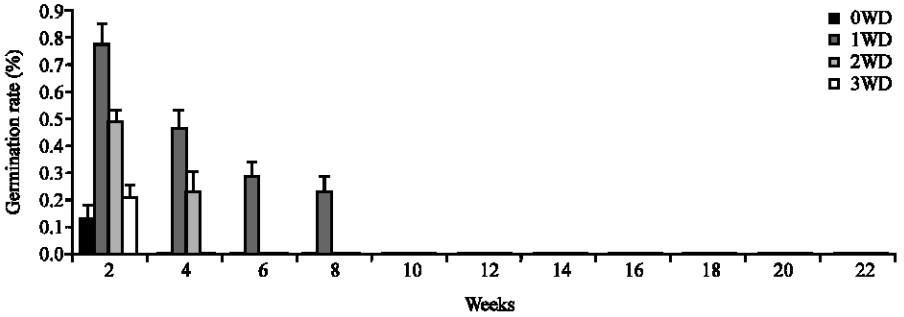

Fig. 7: Influence of drying time (period) on the germinating power of cooled (+ 10°C) *Hymenocardia acida* pollens

of desiccation. This first step of drying restore very low percentages of pollens germination after 4, 6 and 8 weeks (0.46±0.07, 0.29±0.05, 0.23±0.04%) as well extend their critical period of germination after 6 weeks (Fig. 7). The periods of two and three weeks of drying produced less significant effects on the extension of the critical period of storage (2 weeks).

Concerning the effects of initial drying on storage at-20°C, it is clear from Fig. 8 that the water loss of *Callistemon rigidus* pollens during a week does not improve their germination rate after 2 weeks of storage. Notwithstanding, after 4 weeks, the pollens of the first drying period produce a continuous improvement of their germination rate compared to witnesses. The germinating pollens persist among some of this first period of drying after 22 weeks of storage at-20°C (Fig. 8). This increases the critical time storage, raising it from 16 to at least 22 weeks. Of all the periods tested, the initial drying of 2 and 3 weeks did not produce significant positive effect on the storage of pollens. This remark is observed both in the expression of germination of pollens and at the level of persistence over time (Fig. 8). Storage at-20°C in *Hymenocardia acida* shows a weak retention of the germination capacity of dehydrated pollens after 2 weeks (Fig. 9). Only a small amount of pollens in the first period continued to germinate after 8 weeks of storage at-20°C. In general, dehydrated pollens of *Callistemon rigidus* better tolerated freezing than the *Hymenocardia acida* as shown in the storage of pollens from one week of drying (critical period = 22 weeks) (Fig. 8, 9). Moreover, in *Callistemon rigidus*, the 3 sets of dehydrated pollens in addition to the witness have maintained a level of germination after 16 weeks of storage at-20°C.

In both types of storage used (10°C and-20°C), lower germination rate of pollens was very important (> 80%) in both species. However, low levels of germination were better maintained in the pollens of one week of drying after 10 weeks storage and sometimes more at frozen pollens of *Callistemon rigidus* (Fig. 8). The pollens of this species have better germinated and maintained their ability to germinate over time during freezing at-20°C than when refrigerated at 10°C. In *Hymenocardia acida* however, the effects of drying on pollens stored at both temperatures are very similar (Fig. 7, 9).

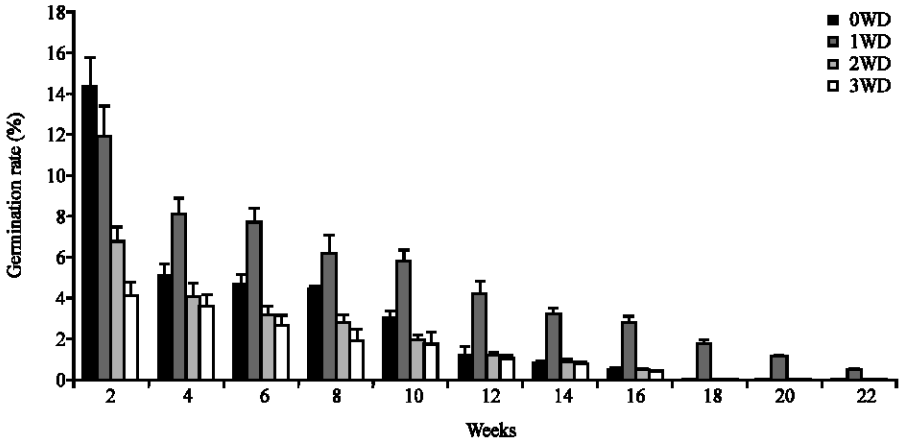

Fig. 8: Influence of drying time (period) on the germinating power of frozen (-20°C) *Callistemon rigidus* pollens

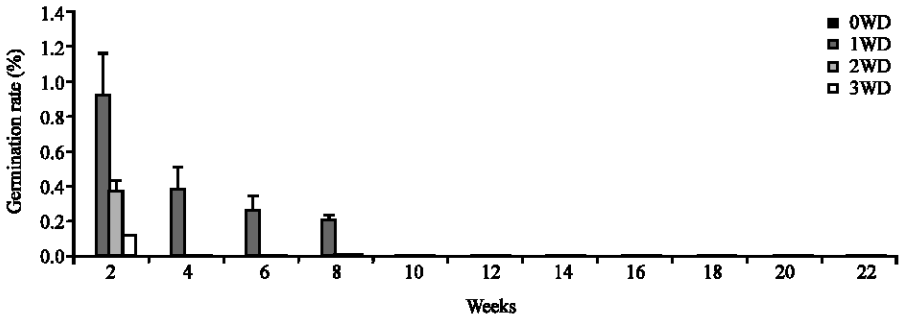

Fig. 9: Influence of drying time (period) on the germinating power of frozen (-20°C) *Hymenocardia acida* pollens

DISCUSSION

The sucrose concentration of medium culture is a factor influencing the germination of pollens of both species and its supply is necessary for their germination (Youmbi, 1993; Heslop-Harrison, 2000). Indeed, in medium lacking sucrose (witness), while pollens of *Callistemon rigidus* have low germination rates, those of *Hymenocardia acida* do not germinate. Similar observations were made by Youmbi (1993) on species like *Pachypodium lamerei*, *Catharanthus roseus*, *Adenium obesum* and Tedjacno (2002) on 3 varieties of *Zea mays* during works which aimed to determine favourable conditions for pollens germination of these species. However, it was noticed that the sucrose concentrations above the optimum can result in dehydration of the cytoplasm and exine structure and inhibit the function of pollen (Visser, 1955; Dumas, 1984; Baloch *et al*., 2001). Similar remarks were made by Mulugeta *et al*. (1994) who studied the dispersion, the viability and the germination of pollens of *Kochia scoporia*, by Baez *et al*. (2002) about the viability and longetivity of pollens of *Nothofagus* species in Southern Chile and Stone *et al*. (2004) during works on the rapid growth of pollen tube in *Conospermum* species. These authors believe in fact that the need for sucrose is limited and a concentration above the optimum would tend to reduce germination capacity. It is known that the physiological process of germination is under the influence of culture temperature. The temperature of 30°C obtained from *Callistemon rigidus* is identical to that of Youmbi (1993) for *Dacryodes edulis* and Dabandata (2000) in oil species *Moringa oleifera* and *Balanites aegyptiaca*. If the optimum temperature of 25°C obtained from *Hymenocardia acida* differs from 30°C in *Callistemon rigidus* and above mentioned species, it is identical to that obtained by Choudhry and Akmedova (1982) in *Gossypium* sp. by Tupy *et al*. (1983) and Ylstra *et al*. (1992) in *Nicotiana tabacum* and Tamnet (2002) in Troncata variety of *Musa acuminata*. The pH of the culture medium also affects the germination of pollen. The difference found between the pH optimum at *Callistemon rigidus* (5) and that of *Hymenocardia acida* (6.8) in this experiment is consistent with the observations of Goddard and Matthews (1981) that the pH value of

germination at first depends to the species. The pH value (5) obtained from *Callistemon rigidus* is similar to that obtained (5,3) by Tamnet (2002) in Pa rayong variety of *Musa acumunata* and corroborates the observations of Holdaway-Clarke *et al.* (2003) which provide a culture medium at acidic pH situated between 5 and 6. Whereas that of *Hymenocardia acida* is outside this range. The low capacity of *Hymenocardia acida* pollens to withstand dehydration beyond the period of 2 weeks shows they are more sensitive to water stress. They may have moisture content higher than that of *Callistemon rigidus* pollens. Indeed, it is demonstrated that the pollens that have a high moisture content at anthesis (> 40%) are sensitive to stress (Digonnet-Kerhoas and Gay, 1990) and in particular to dehydration. In this species where the pollens contain some characters belonging to anemophilous pollens (smooth exine for example), their viability drops more quickly (Cerceau-Larrival, 1988). The low viability could also be due to a change in the composition of the exine related to glycoproteins, glycolipids and carotenoid pigments (Cerceau-Larrival, 1990). The gradual decline in the germination of *Callistemon rigidus* pollens over time was significantly longer (spread over 6 weeks). The best resistance of pollens of this Myrtacea to water stress is consistent with the findings of Heslop-Harrison (2000) in another Myrtacea: *Eucalyptus rodantha*, where it was raised pollen adaptations during drying at 70°C allowing survival for several months. By cons, our results are significantly different from those of Tedjacno (2002) in which the germination of pollens in 3 varieties of *Zea mays* couldn't tolerate dehydration over a period exceeding 2 hours. The non-dehydrated pollens (controls) in both species can be kept shorter in the refrigerator (10°C). Dehydrated pollens of *Callistemon rigidus* species better withstand storage at 10°C and-20°C while those of *Hymenocardia acida* quickly lose their ability to germinate. In addition, the duration of a week of drying has a considerable extension of the critical period of storage of pollen at-20°C in *Callistemon rigidus* (over 22 weeks). This result is lower compared to that of Farcy *et al.* (1990) in *Petunia hybrida* where the critical period of storage was greater than 50 weeks, Youmbi *et al.* (1998) in *Dacryodes edulis* with a critical period exceeding 100 weeks and Siregar and Sweet (2000) in *Radiata* pine where the critical period was over 50 weeks. On the other hand, it was not possible during a similar experiment bearing on the germination capacity of pollens stored at +10°C and -20°C to have a critical period exceeding the duration of 5 h in varieties of *Zea mays* (Tedjacno, 2002) where, the pollen was very sensitive to water stress. The positive effect of the one week period of drying could reduce the irreversible loss of the permeability of the plasma membrane of pollen detrimental to germination mechanism. Emphasis on the recalcitrant nature expressed by non-dehydrated *Hymenocardia acida* pollens during storage at-20°C is due to their high humidity. It is indeed demonstrated that in these types of pollen, freezing causes the crystallization of the intracellular water which is not evacuated because they suffered no initial drying (Mazur, 1984). This will effectively reduce or inhibit their germination.

It definitely appears that the BK culture medium enriched with sugar concentration of 10 to 15% and set to culture room at 25 or 30°C allows a better expression of pollen germination. Time for a week of drying significantly extended the critical period of storage of pollens at +10 and -20°C for both species. It seems important in future work to extend the germination tests beyond 22 weeks in the *Callistemon rigidus* species and expand this work to others bee species threatened in this region of Cameroon.

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