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OIL PALM (*Elaeis guineensis* Jacq.) IMPROVEMENT: POLLEN ASSESSMENT FOR BETTER CONSERVATION AND GERMINATION

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ABSTRACT

Planting of improved Elaeis guineensis *seeds to increase oil production is a sustainable strategy to mitigate the impact of crop-based demand for bioenergy. But breeders need a quality pollen stock for successful hybridisation of outcrossing plants. This study was carried out to determine suitable conditions for* in vitro *germination and conservation of oil palm pollen. Performance of pollen germination was measured in three distinct culture media: Brewbaker and Kwack (BK), Heslop-Harrison (HH), and Arnaud (FA) media, each supplemented with seven sucrose concentration [suc] (0%, 5%, 10%, 15%, 20%, 25%, 30%) for five incubation durations (1-5 hr). Optimal germination scores of 73%, 41% and 39% for BK (15% [suc]), HH and FA (10% [suc]) were obtained respectively after 3 hr of incubation at 38°C. Fresh pollen was also treated to four moisture content (MC) ranges: 100%, 78%-70%, 65%-57% and 41%-3% [on fresh weight (FW) basis].* In vitro *germination scores of 81%, 83%, 56% and 29% were obtained respectively when cultured in BK (15% [suc]) after 3 hr of incubation. After 50 days of storage, pollen with MC 78%-70% scored* in vitro *germination of 70% and 3% at -20°C and 10°C, respectively. These results precisely determine optimal conditions for oil palm pollen* ex situ *conservation thereby optimising its usage.*

Keywords: *Elaeis guineensis*, pollen, conservation, culture media, *in vitro* germination.

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INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is a perennial monocot of the *Arecaceae* family (Castilho *et al*., 2000). It is the most important and the highest oil yielding tropical crop in the world (Corley *et al*., 2003) with world average oil yield of 3.5 t of palm oil per hectare per year (Seegräf *et al*., 2010). Oil palm has become the main oleaginous plant compared with other major oil crops like rapeseed, soyabean and sunflower (Charrier *et al*., 1997). The crop is grown on a large scale in Africa, Equatorial America, South-east Asia and South of the Pacific, and in smaller scales in other parts of the world. Oils obtained from its fruits are crude palm oil extracted from the mesocarp and palm kernel oil extracted from the kernel (Hardter and Fairhurst, 2003). Palm oil presently occupies the first position among all dietary vegetable oils, with an annual global production equating to about 39% of the world production of vegetable oils (USDA, 2012).

The present consumption rate of palm oil both in the food and the manufacturing industries, coupled with its intensive recent exploitation as biofuel (Frank *et al*., 2009), calls for an increase in production for fear that demand outweighs supply. Increasing the sizes of plantations alone is

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not a solution enough to mitigate future demand and supply, hence, synchronising the latter with improved planting material could be a better option. Like other allogamous plant species, the production of improved oil palm seedlings involves controlled pollination between parents with interesting complementary traits. To this effect, there is a need for proper handling of pollen from the field to the laboratory where it is conserved for subsequent use.

Controlled pollination is the ultimate answer to production and valorisation of improved oil palm seedlings given that natural hybridisation in allogamous plant species is generally associated with shortcomings like: short life-span of pollen, loss of an important quantity of pollen, poor synchronisation between pollen shed and stigma receptivity (Youmbi *et al*., 2012), and above all, the production of hybrids with doubtful yields.

International companies are increasingly buying land for the establishment of oil palm plantations (Hoyle and Levang, 2012) for biofuel production and food. These huge upcoming planting projects will require considerable amounts of improved *Tenera* planting materials and relevant amounts of pollen to be produced locally or imported in order to meet these needs. It is therefore important to have a mastery of *ex situ* parameters that affect pollen conservation, as well as to identify appropriate techniques for testing pollen viability. Previous studies have provided full handbooks on pollen harvesting, conditioning, conservation and viability check (Hardon and Davies, 1969; Turner and Gillbanks, 1974; Ekaratne and Senathirajah, 1983). However, these procedures may be significantly impacted by many factors like the changing environment with increasing temperature, progenies and other technical parameters (Myint *et al*., 2012).

The goal of this study was to determine the optimal conditions at which freshly collected oil palm pollen could be conserved *ex situ* thereby maximising its duration of conservation and usage. To reach this goal, the following objectives were studied: (1) determination of culture medium, sucrose concentration and incubation duration that optimise germination of oil palm pollen *in vitro*, and (2) a comparative study on the effect of: (a) four endogenous moisture content (MC) ranges, and (b) three temperature regimes for conservation.

METHODS

Pollen used in this study was collected from the three varieties of oil palm, *i.e. Dura* (D) (identification N° B71 Po 6850-02-03 and 07-01), *Pisifera* (P) (identification N° C17-Lm 18978-34-30 and A79-Lm 18228-08-19) and *Tenera* (T) (identification N° A79-Lm18228-08-26 and 08-21) all grown in the Specialised Centre for Oil Palm Research (CEREPAH) IRAD, Cameroon.

Pollen Collection, Processing and Assessment of Germination *in vitro*

Immature male inflorescences were identified and isolated using specialised bags designed for that purpose. Nine days after isolation, daily visitations were made. Once two-thirds of an inflorescence attained anthesis, it was harvested, still in the pollination bag, and taken to the pollen laboratory. The harvested inflorescences were dried at room temperature (30°C) and humidity (30% - 40%) for 2 hr. Threshing was done in a previously sterilised isolation box followed by sieving with a sterilised metal sieve. The pollen was then put in special plastic bags, locked up in a 2-litre container half filled with ice blocks and transported to the plant improvement unit of the laboratory of environment and biotechnology of the University of Yaounde I, where optimum *in vitro* germination and storage conditions were to be determined.

Once pollen arrived at the laboratory, the viability of the fresh pollen was assessed in the three culture media, prepared according to Arnaud (FA) (1979), Brewbaker and Kwack (BK) (1963) and Heslop-Harrison (HH) (1979). The experimental design was completely randomised in a 3 * 3 * 5 * 7 * 3 factorial scheme including three oil palm varieties, three culture media, five incubation periods, seven sucrose concentrations [suc] and three repetitions.

For each treatment, a drop of culture medium was put on a labelled glass slide and pollen was homogenously sown on the slide in triplicates. The latter were then put in saturated atmosphere Petri dish and incubated at 38°C. After every hour the triplicate for each culture medium were removed, and observed under a photon microscope model CH, Olympus optical Co., Ltd, and the germination (*Figure 1*) rate scored at 100X. Random counts of 250 - 300 pollen grains were made in three different regions per slide and germination rate expressed in percentage. For each sample, five observations corresponding to hourly incubation intervals were made. The percentage of germination was calculated as described by Arnaud (1979).

Measurement of MC, Packaging and Conservation

Triplicates of 4 g of fresh pollen were desiccated for one, two, three and four days representing four desiccation periods respectively. At the end of each desiccation period, the three repetitions for the said duration were removed from the desiccator, reweighed using the same electronic balance and the dry weight (DW) of each repetition recorded. The MC of each repetition was calculated as follows; let A be the weight of the empty Petri dish, B the weight

of the Petri dish containing the fresh pollen and C the weight of the same Petri dish containing dried pollen. The MC in percentage was given as $\frac{B - C}{C - A} * 100$ (Arnaud, 1979).

After measuring the dry weight, 1 g of homogenously mixed pollen from repetitions with minimal variation in MC was filled in vials in triplicates and respectively conserved at ambient temperature (AT), 10°C and -20°C representing the three temperature regimes under test. Fresh pollen (MC taken as 100%) served as the control and was equally stored at the different temperature regimes. *In vitro* germination assessment of dried pollen conserved at 10°C and -20°C was conducted on intervals of 10 days, while for non-desiccated pollen, germination was assessed after every five days. For pollen conserved at AT, with and without desiccation, germination tests were conducted on a daily basis.

The experimental design in this second part was also completely randomised into a 4 * 3 * 3 factorial scheme including four MC ranges (100%, 78%-70%, 67%-57%, and 41%-31%), three temperature regimes (10°C, -20°C and AT) for conservation and three ages (7, 14 and 18 years old palms). The methodology was adapted from those proposed by Arnaud (1979) and Youmbi *et al*. (2005).

Statistical Analysis

Analysis of variance (ANOVA) was done using two-way Anova and the levels of significance determined using the Student-Newman-Keuls Multiple Comparisons Test and the Bonferroni post-test. Standard errors are provided where necessary.

RESULTS

Effects of Culture Medium and Sucrose Concentration [suc] on *in vitro* Germination

Preliminary *in vitro* germination tests carried out on fresh pollen showed that pollen from the three varieties of oil palm germinated in all the three culture media, (FA, HH, and BK) but there were statistically significant differences ($p < 0.001$) in the rate of *in vitro* germination with [suc] within and between the culture media (two-way ANOVA). A pollen grain was considered to have germinated (*Figure 1*), when the length of the pollen tube was twice the diameter of the pollen grain (Dafni, 2000). Photographs of germinated pollen (*Figure 1*) were taken directly from the Olympus Optical Co., Ltd microscope.

The study of the effect of [suc] within each base medium (*Figure 2*) showed that the rate of *in vitro* germination increased with increase in sugar

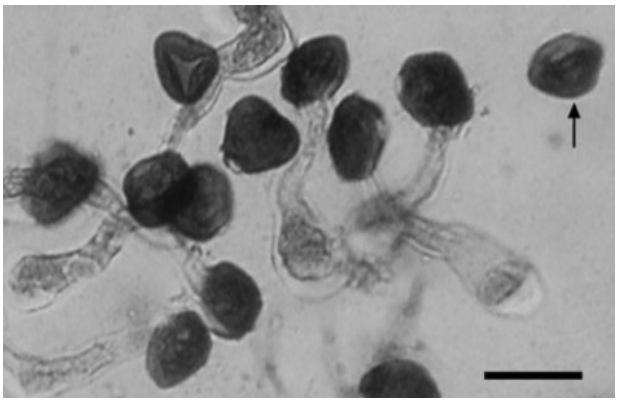

Figure 1. Germinated pollen grains with long pollen tubes and non-germinated pollen. Bar = 8 µm, arrow indicates ungerminated pollen.

concentration up to a certain threshold where germination rate dropped drastically. On Brewbaker and Kwack (BK) medium, the highest germination was recorded at a [suc] of 15% w/v with a score of 75%, while the least germination of 0% was observed at a [suc] of 0% w/v. On Heslop-Harrison (HH) medium, a maximum germination score of 46% was obtained at a [suc] of 10% w/v while the least rate was noticed in 0%. As for FA medium, the highest germination score was 45% at a [suc] of 10% w/v, with the least germination rate of 1% recorded in a [suc] of 30% w/v (*Figure 2*). Variance analysis showed that there was no significant difference in pollen germination rate between [suc] of 0%, 20%, 25% and 30% w/v in all culture media at $P > 0.05$ (by Student-Newman-Keuls Multiple Comparisons Test). On the other hand, highly significant differences were recorded at $p < 0.001$ between [suc] of 5%, 10% and 15% w/v and 0%, 20%, 25% and 30% w/v in all culture media.

The [suc] at which optimal germination occurred in the three media was not the same, indicating that each medium had a [suc] at which pollen germination was at maximum.

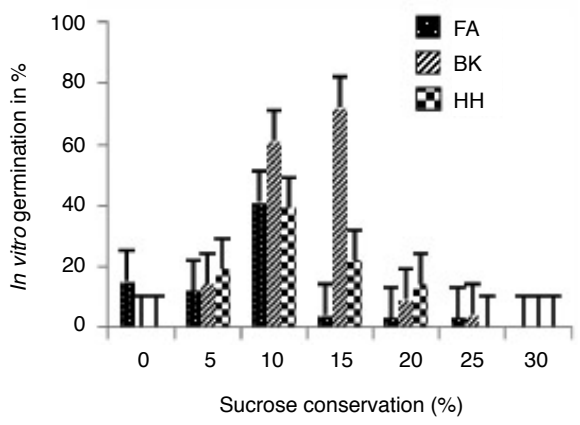

Figure 2. Effects of sucrose concentration [suc] in culture medium on in vitro *germination.*
HH - Heslop-Harrison medium, BK - Brewbaker and Kwack medium, FA - F. Arnaud medium.

Influence of Incubation Duration

For incubation durations of 1, 2, 3, 4 and 5 hr, germination was observed respectively in the three culture media as follows: in HH medium +10% [suc] the average germination scores were 6%, 26%, 41%, 41%, and 41% (*Figure 3a*); in BK medium + 15% [suc], corresponding average germination rates of 26%, 51%, 64%, 65% and 65% were scored (*Figure 3b*); while in the FA medium + 10% [suc], 8%, 27%, 39%, 39% and 39% in vitro germination rates were respectively recorded (*Figure 3c*).

Highly significant differences ($p < 0.001$) in the rate of *in vitro* germination were obtained between first, second and third hour of incubation, while from the third, fourth and fifth hour, no significant differences were found in all culture media, and in all the three varieties. This reveals that 3 hr of incubation is enough to obtain peak germination rates of oil palm pollen *in vitro*. According to the results, significant variations were observed in germination of pollen collected from *Dura*, *Pisifera* and *Tenera* in all base media and incubation time, with *Pisifera* pollen registering the best average rate of germination (*Table I*).

Effect of MC on *in vitro* Germination of Pollen Grains

As for pollen that underwent desiccation, pollen with MC range of 78%-70% recorded the highest germination score of 82.7%, followed by 56.3% for MC of 67%-57%and 29% for MC of 41%-31% (*Figure 4*). The interaction, MC x age of pollen source x conservation temperature showed highly significant differences at $P < 0.001$ (two-way ANOVA). Statistically, no significant differences were recorded between MC of 100% and 78%-70% in pollen collected from oil palms of 7, 14 and 18 years while highly significant differences were noticed between MC ranges of 78%-70%, 67%-57% and 41%-31% (Bonferroni post-test).

Effect of Temperature Regime of Conservation on *in vitro* Germination of Pollen

As per the effect of temperature regime for storage, after 50 days, pollen with MC range of 78%-70% stored at 20°C and 10°C respectively recorded viability losses of 6.6% and 97% (*Figure 5a*). Germination was zero after 20 days for non-desiccated pollen in all three storage temperature regimes (*Figure 5b*). Statistically, highly significant differences were obtained at $p < 0.001$ (two-way ANOVA) among the temperature regimes at which pollen was preserved as well as for the interaction, method of conservation x duration of conservation.

DISCUSSION

To obtain reliable estimates of pollen viability *in vitro*, a culture medium that favours optimum expression of physiological capacity for pollen tube formation and growth is needed (Soares *et al*., 2008). In this study, optimal in vitro germination

TABLE 1. EFFECT OF CULTURE MEDIUM AND INCUBATION TIME ON POLLEN GERMINATION OF DIFFERENT OIL PALM VARIETIES

Culture medium	Variety	Incubation time (hr)				
		1	2	3	4	5
BK, 15% [suc]	D	19.7±0.00^{a}	44.7±2.02^{a}	57.0±3.00^{a}	58.0±2.02^{a}	58.3±2.60^{a}
	P	29.7±2.02^{b}	61.3±3.48^{b}	72.0±1.16^{b}	72.7±1.45^{b}	72.7±1.45^{b}
	T	19.0±0.33^{a}	47.0±0.58^{a}	63.3±1.45^{a}	63.7±1.45^{a}	63.7±1.45^{a}
HH, 10% [suc]	D	6.6±0.33^{a}	19.7±0.33^{b}	40.3±0.88^{b}	40.7±0.88^{b}	40.7±0.88^{b}
	P	4.7±0.33^{b}	26.7±2.02^{ab}	39.0±0.50^{ab}	39.0±0.58^{ab}	39.0±0.58^{ab}
	T	7.0±1.16^{a}	30.7±2.62^{a}	43.0±1.15^{a}	43.7±1.45^{a}	43.7±1.45^{a}
FA, 10% [suc]	D	4.0±0.57^{a}	25.0±1.15^{a}	37.0±1.12^{a}	37.0±1.16^{a}	37.0±1.16^{a}
	P	9.0±1.15^{b}	29.0±0.58^{b}	41.0±0.58^{b}	41.0±0.58^{b}	41.7±0.33^{b}
	T	9.7±0.33^{b}	28.0±4.04^{b}	38.0±4.04^{ab}	39.0±3.46^{b}	39.7±3.18^{b}

Note: Values are means ± standard error. Means of pollen germination within columns followed by different letters are significantly different at 5% level, according to the Bonferroni post-comparison test. D - *Dura*, P - *Pisifera*, T - *Tenera*, HH - Heslop-Harrison medium, BK - Brewbaker and Kwack medium, FA - F. Arnaud medium. [suc] - sucrose concentration.

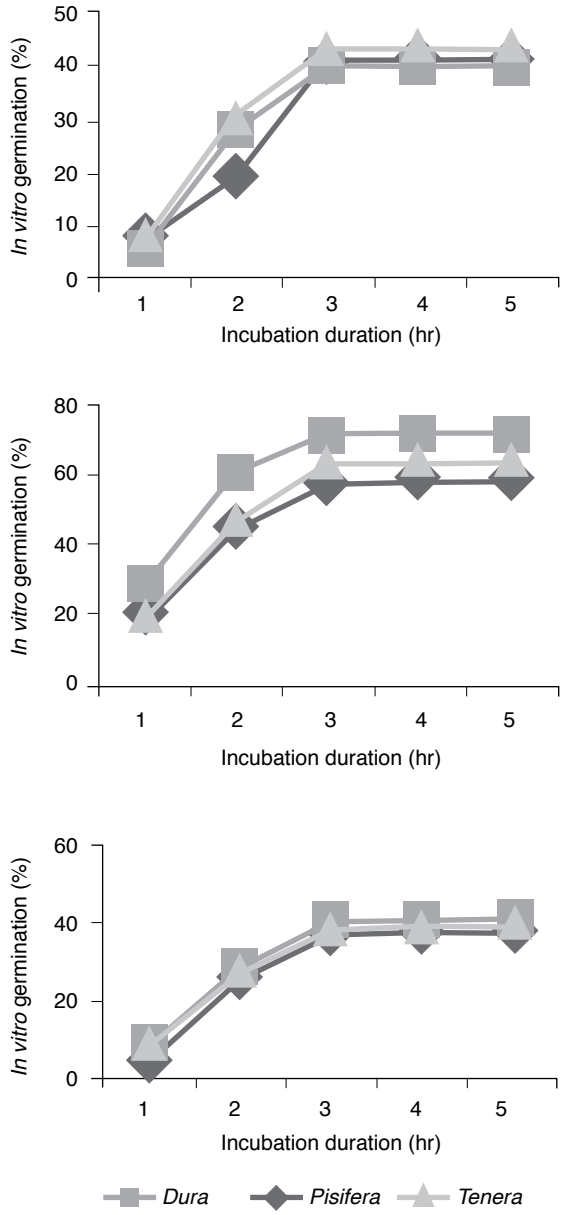

Figure 3. Effect of incubation duration on pollen germination of the three varieties in the three base media; a) Heslop-Harrison (HH) medium with 10% sucrose concentration [suc]; b) Brewbaker and Kwack (BK) medium with 15 % [suc]; c) F. Arnaud (FA) medium with 10% [suc].

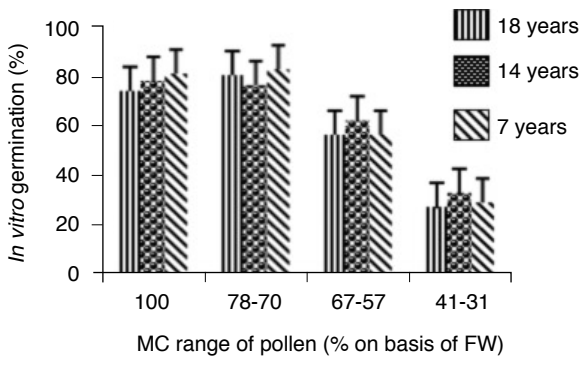

Figure 4. Effect of moisture content (MC) on rate of in vitro *germination of pollen collected from oil palms of different ages.*

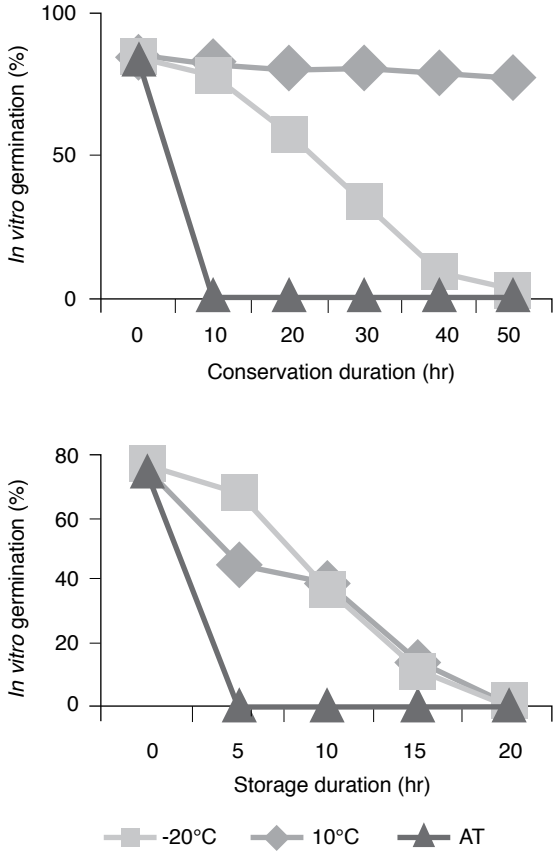

Figure 5. Effect of storage temperature on in vitro *germination of a) desiccated pollen with 78%-70% moisture content and b) non-desiccated pollen with 100% moisture content. AT-ambient temperature.*

in the three culture media was obtained after 3 hr incubation. This corroborates with the high average percentage of *in vitro* germination of feijoa [*Acca sellowiana* (Berg) Burret] obtained after 3 hr of incubation, but at 25°C (Frazon *et al*., 2005).

The results also show that each base medium has a threshold sugar concentration beyond which *in vitro* germination is negatively affected. In BK, [suc] threshold is 15%, while for F. Arnaud (FA) and HH media, optimal [suc] is 10%. The present result confirms the hypothesis which states that an increase in [suc] beyond the limit of a given species alters the osmotic potential of the medium, causing inhibition of pollen tube formation *in vitro* (Premanchandra *et al*., 1992). The energy required for the germination of pollen grains, formation of cell wall components and callose in angiosperms is provided by the nutrient reserves stored in the pollen grains (Youmbi e*t al*., 2012); the role of sugar is merely for osmotic balance. The difference in pollen germination within the same culture medium with different [suc] observed in this study could therefore be attributed to a difference in osmotic pressure of the culture media.

The [suc] [15% (w/v)] and incubation duration (3 hr), being constant in the three culture media, higher germination in BK than FA and HH is

probably due to the presence of oligominerals like calcium nitrate [$Ca(NO_3)_2H_2O$], boric acid (H_3BO_3), potassium nitrate (KNO_3), and magnesium sulphate ($MgSO_4 7H_2O$) in BK medium. Even though the HH medium also contains two (boric acid and calcium nitrate) of the four germination stimulating micronutrients in BK, its germination percentage remained lower than on BK medium at all [suc]. These results are contrary to those obtained with maize pollen which demonstrated that HH medium had higher pollen germination than BK (Youmbi *et al*., 2005). Each of these micronutrients has been reported to play a specific role required to trigger pollen germination *in vitro* (Premanchandra *et al*., 1992). Boric acid is involved in the formation of a sugar-borate complex that ionises rapidly with the cell membrane thereby facilitating the absorption, translocation and metabolism of molecules of sugar in pollen (Souza-Lang and Pinto, 1997). Boron is believed to promote pollen germination by affecting H^+-ATPase activity, which initiates pollen germination and tube growth (Feijó *et al*., 1995).

The result of the present study equally indicated that pollen collected from the three varieties of oil palm (D, P and T) showed significant differences in different culture media and incubation time (*Table I*). These results confirm those of a recent study in which significant variations were observed upon assessing pollen germination of the three varieties of oil palm (D, P and T) in solid and liquid sucrose and glucose media (Myint *et al*., 2012). However, in the present study, *Pisifera* pollen gave the highest germination rather than *Tenera* pollen in a recent study (Myint *et al*., 2012). This behaviour ties with previous studies which reported that there are differences between species and cultivars of a given species in terms of conditions required of a medium for optimal expression of pollen germination potentials *in vitro* (Frazon *et al*., 2005).

As far as the conservation of pollen is concerned, MC and temperature are the two main critical external factors that affect the maintenance of pollen germination capacity (Khan and Anjum, 2009). It has been reported that spells of drought and high temperature are implicated in the release of bad-quality pollen under natural conditions (Schoper *et al*., 1987). The effect of endogenous MC of pollen on in vitro germination was part of this study. No significant difference was noticed in germination of pollen that did not undergo any prior desiccation (MC considered to be 100%) and pollen desiccated to MC of 78%-70% (on FW basis). Pollen with MC < 70% scored low percentages of germination.

The rate of pollen germination decreases with a decrease in MC (*Figure 4*). The present results tie with an earlier study which found that at 30% MC, somatic tissues irreversibly lost their viability, and below 30% MC, membranes and many enzymes undergo conformational changes which lead to loss of integrity and inactivation (Crowe *et al*., 1986). This could be a possible reason why in the present study a MC range of 41%-31% gave the least pollen germination percentage (29.6%) compared to 79.7% and 58.2% germination for MC ranges of 78%-70% and 67%-57%, respectively.

Concerning the temperature regime for pollen storage (*Figure 5*), results obtained in this study showed that for the 50 days of pollen conservation, viability loss of only 6.6% occurred for pollen dried to MC of 78%-70% and conserved at -20°C, while viability loss of 96.1% was recorded for pollen with the same MC but conserved at 10°C. However, pollen with a MC of 78%-70% conserved at 10°C maintained an interesting viability of 79.3% after 10 days, indicating that 10°C could be used for short-term preservation of pollen with MC of 78%-70% without fear of any significant loss in viability. This result coincides with those found with pollen of three varieties of date palm (*Phoenix dactylifera*) in which an *in vitro* pollen germination of 78% was reported after 40 days of deep freezer storage at -20°C (Mortazavi *et al*., 2010). Pollen stored at -20°C has been reported to maintain viability for more than a year by conditioning in vacuum sealed vials (Bénard and Noiret, 1969; Ichikawa and Shiden, 1971).

The high viability loss in pollen stored without prior desiccation (*Figure 5b*) may be attributed to the formation of ice inside the cytoplasm due to much free endogenous water in the cytoplasm. Ice formation in the cytoplasm had been reported not only to denature the activities of enzymes that trigger pollen germination, but is also injurious to the cell and membrane bound organelles (Youmbi *et al*., 2012).

In this study, non-desiccated pollen (MC=100%) conserved at AT could still show some viability for up to five days (*Figure 5b*). These results are contrary to those reported in maize pollen which had a life-span of around 24 hr when stored under AT (Youmbi *et al*., 2005). The longevity of oil palm pollen for up to five days at AT could be explained by the fact that oil palm pollen is bicellular and so undergoes natural dormancy after pollen shed (Barcellona *et al*., 2004). This natural dormancy is an adaptation to rapid dehydration accounting for an additional advantage for long-term conservation of plants with bicellular pollen than plants with tricellular pollen like maize (Youmbi *et al*., 2005).

CONCLUSION

In conclusion, this study illustrates that optimal germination of fresh and dehydrated oil palm pollen could be obtained *in vitro*, by sowing pollen in BK medium containing 15% w/v [suc], and incubated for 3 hr at 38°C±1°C. Secondly, conservation of oil

palm pollen could be maximised by desiccating pollen to a MC of 78 ≥ 70% (on FW basis) prior to storage. Long-term storage (≥ 50 days) can be achieved at -20°C while short-term (≤ 10 days) storage can be attained at 10°C without any significant loss in viability. Finally, the age of oil palm from which pollen to be used for hybridisation is collected, does not influence pollen germination *in vitro*. The present study has identified a MC range and temperature regime that optimise conservation of oil palm pollen and a suitable method to test viability of conserved pollen. These results fall in line with the interest of prolonging the life-span of biological material *ex situ*, thus possibility to be used when and where needed.

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