MORPHOLOGICAL AND *IN VITRO* GERMINATION STUDIES OF POLLEN GRAINS IN KOLA TREE (*COLA* SP.)

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Abstract

This study was carried out to determine some morphological characteristics and *in vitro* germination capacities of pollen grains of three cultivated (*C. ballayi*, *C. acuminata* and *C. anomala*) and one wild (*C. lepidota*) species of *Cola* genus. Results indicated that pollen grains were morphologically tricolporate, isopolar, subcircular, and prolate with a smooth exine outer layer. *In vitro* germination was optimal in Brewbaker medium containing 5 % sucrose for *C. acuminata* and *C. lepidota*, 10 % for *C. anomala* and 5 or 10 % sucrose for *C. ballayi*. Higher germination rates were obtained at 30°C incubation temperature for all species. Germination rates of *C. ballayi, C. acuminata, C. anomala* and *C. lepidota* peaked at pH 5.1, 5.7, 6 and 6.3, respectively. Incubation beyond seven hours was necessary for optimal germination and pollen tube elongation.

Key words: *Cola*, pollen, morphology, *in vitro* germination, pollen tube elongation.

Kola (*Cola* sp.) Ağacı Polenlerinin Mofolojik Özellikleri ve *In Vitro* Çimlenmeleri Üzerinde Araştırmalar

Özet

Bu çalışma, *Cola* cinsine ait üç kültüre alınmış (*C. ballayi*, *C. acuminata* ve *C. anomala*) tür ile bir doğal türün ((*C. lepidota*) polenlerinin bazı morfolojik özellikleri ve *in vitro* çimlenme kapasitelerinin belirlenmesi amacıyla gerçekleştirilmiştir. Sonuçlar polenlerin morfolojik olarak tricolporate, isopolar, subsircular ve daha dış tabaklarda düz exine ile prolate olduğunu ortaya koymuştur. Brewbaker ortamında optimal çimlenme için uygun şeker (sakaroz) oranı *C. acuminata* ve *C. lepidota* türleri için %5, *C. anomala* türü için %10, *C. ballayi* türü için ise %5 veya %10 olarak saptanmıştır. Tüm türlerde 30°C inkübasyon sıcaklığında daha yüksek çimlenme oranları elde edilmiş ve *C. ballayi, C. acuminata, C. anomala* and *C. lepidota* türlerinde çimlenme oranları sırasıyla 5,1, 5,7, 6,0 ve 6,3 pH değerlerinde en yüksek noktaya ulaşmıştır. Tüm türlerde optimum çimlenme ve polen tüpü uzaması için yedi saatten fazla süreye gerek olduğu belirlenmiştir.

Anahtar Kelimeler: *Cola*, Polen, Morfoloji, *In Vitro* Çimlenme, Polen Tüpü Uzaması

1. Introduction

The kola tree, family *Sterculiaceae*, is a woody plant from Tropical Africa and is cultivated for wood and seeds which contain stimulating products (Tindall, 1998). Its seeds belong to Non Timber Forest Products (NTFP). Seed harvesting and sales represent about 5 to 37 % of inhabitant income in the West Cameroon (Laird et al. 1997). *Cola* had been the subject of few works and scientific research on the domestication of kola tree in Central and West Africa is still in infancy (Tchoundjeu et al., 1998). Studies on the biological flower revealed the presence of hermaphrodite and male flowers (Nkongmeneck, 1985) which are auto-incompatible or auto-compatible (Anonymous, 1993). During a year, flowering of kola tree is characterised by the production of male flowers, hermaphrodite flowers and male flowers successively (Anonymous, 2002). Major problems encountered during the exploitation of the *Cola* spp. included conservation, seed quality and biological factors (diseases,

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precocious dropping of fruits and the lower presence of hermaphrodite flowers). This study is part of the preliminary work on kola pollen grains. The results obtained would allow to facilitate hybridization between plants that flower at different times (Nkongmeneck, 1982). *In vitro* germination is one of the most convenient and reliable methods to evaluate the viability of fresh and stored pollen (Jayaprakash and Sarla, 2001). The main purpose of this study was to determine the morphological characteristics and *in vitro* conditions for optimum germination of *Cola* pollen grains.

2. Material and methods

2.1. Plant materials

Kola trees in Cameroon are located in South of 8th parallel (Nkongmeneck, 1982). The pollen grains used in this study were freshly collected from four *Cola* species. These were: three cultivated species (*C. ballayi* and *C. acuminata* at Yaounde in the Centre province; *C. anomala* at Dschang in the West) and one wild species (*C. lepidota*) at Mutenguene in Sud-West Province.

2.2. Morphology

Morphological characteristics of pollen grains were determined using acetolyse technique (Erdtman, 1952). Pollen grains were soaked in a acetolysing solution made up of acetic anhydride and concentrated sulphuric acid (9:1 v/v), kept into cold bain-marie which was allow to boil for one minute and then cooled. The mixture was centrifuged at 1800 t/min for ten minutes. Two rinsings per centrifugation using acetic acid and two others with distilled water were done. The pollen grains were then soaked in a solution of glycerine water (50:50 v/v) for thirty minutes followed with a last centrifugation. Tubes were then turned down to obtain dehydrated pollen grains. For observations under light microscope (Olympus CH-2), pollen was mounted on slides containing some drops of solution from a mixture of gelatin and glycerin (50:50 v/v) and was then covered with cover glass attached on the slide with paraffin. Diameter and the pollen tube length were measured using the micrometer of the microscope.

2.3. In vitro germination

Two classical culture media were used to measure the *in vitro* pollen germination: Brewbaker & Kwack (1963) and Heslop-Harrison (1979) media. The medium that permits an optimal germination was determined using freshly harvested pollen from the 4 *Cola* species. Eight saccharose concentrations (0, 5, 10, 15, 20, 25, 30 and 35%), five temperature values (25, 30, 35, 40 and 45°C) and nine pH values (4.5, 4.8, 5.1, 5.4, 5.7, 6.0, 6.3, 6.6 and 6.9) were investigated. The pH was measure using SCHOT GERATE 818 pH metre.

The two germination media prepared were mounted on slides and spread with pollen grains using a magnifying glass. These slides were stored in Petri-dishes under saturated atmosphere (the saturation of the atmosphere was obtained by adding water on Watman paper) and incubated at 30°C constant temperature for 24 hours. The germination rate of the pollen was determined after 24 hours. Slides removed from the incubator were stained using a method described by Alexander (1969) and a Photon microscope was used to count the number of germinated pollen. A pollen grain was considered as germinated if the tube length was bigger than the diameter of pollen grain (Shivanna and Rangaswamy, 1992). Pollen tube length and germination kinetic were evaluated from 1 to 7 hours.

Germination data was collected on a sample of at least 600 pollen grains on a field of the slide in a split plot design with three replications for each medium treatment. Similarly, 60 pollen tubes chosen randomly in each treatment were measured to calculate mean pollen tube length. Germination rates were used to determine the best culture medium and the optimum saccharose, temperature and pH values. Statistical analysis was made using SAS GLM procedure (SAS, 2001).

3. Results

The pollen grains of four *Cola* species were isopolar, longiaxe, subcircular and tricolporate (Fig. 1A). Measurements indicated that *C. lepidota* showed a polar diameter of 49.62µm, equatorial diameter E: 43.92 µm and a polar to equatorial (P/E) ratio of 1.12 and possessed the biggest pollen grains. Pollen grains of *C. acuminata* were smallest and possessed a polar diameter of 40.88 µm, an equatorial diameter 30.87 µm and highest P/E ratio 1.41. *C. acuminata* had a thicker outer exine layer and was followed by *C. lepidota, C. ballayi* and *C. anomala* (Table 1). The external exine surface of *Cola* pollen grain is smooth.

Fresh pollen grains of all *Cola* genus tested, germinated in both classical basal media: Brewbaker and Kwack (BK), and Heslop-Harrison (HH). For all varieties tested, the germination rates were higher on BK medium than on HH medium (Fig. 1B). On BK medium, the optimum germination rate of *Cola ballayi* was 64.65.± 3.76 % whereas it was 45.54 ± 3.54 % on HH medium (Fig. 2A). The effect of the saccharose level supplemented in the base medium showed that germination rate increased with the increase in saccharose concentrations with the optimum at 5 or 10 %, and 15 % on BK and HH base media respectively. Above these saccharose levels, germination rates decreased gradually and became zero at 35 % saccharose (Fig. 2A). The statistical analysis revealed higher significant differences between saccharose concentrations (Table 2).

The effect of base media and saccharose concentrations on pollen grains germination of *Cola acuminata* showed that the higher germination rate was 58.08 ± 5.86 % on Brewbaker and Kwack medium supplemented with 5 % saccharose concentration (Fig. 2B). Pollen germination rate was lower (39.24 ± 2.24 %) on Heslop-Harrison with 15 % saccharose concentration. At 35 % of saccharose concentration, no germination rate was observed in both the media. Statistical analysis showed higher significant differences between saccharose concentrations ($p \leq 0.001$), and base media ($p \leq 0.01$) (Table 2).

The *in vitro* germination rates of *Cola anomala* varied according to the medium and the saccharose concentration (Fig. 2C). On BK medium without saccharose, the germination rate was lower (0.34 ± 0.09 %).

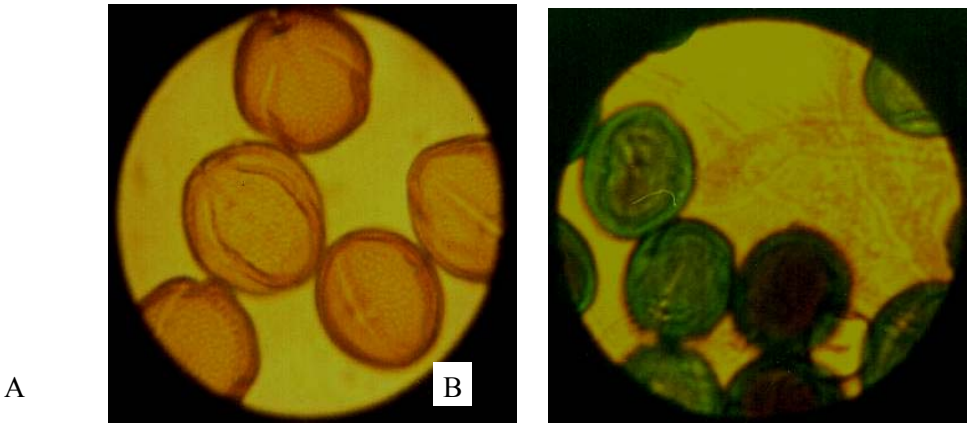

Figure 1. *Cola* pollen grains. A: acetolysed pollen, B: group of germinated pollen.

Table 1. Dimensions of pollen grains in four *Cola* species.

	Dimension (mean±SD)			
Species	Polar length (P) (µm)	Equatorial length (E) (µm)	P/E	Exine (µm)
C. ballayi	39,78 ±2,37	33,75 ±2,00	1,17	3,43 ±1,00
C. acuminata	40,88 ±2,31	30,87 ±2,43	1,41	3,97 ±1,05
C. anomala	39,71 ±2,40	33,02 ±2,64	1,20	3,34 ±0,77
C. lepidota	49,62 ±7,40	43,92 ±4,42	1,12	3,67 ±0,80

Table 2. F values from ANOVA for the effect of culture medium and saccharose concentration on *Cola* pollen grains germination.

		Species			
Source of variation	df	*C. ballayi*	*C. acuminata*	*C. anomala*	*C. lepidota*
Block	2	0.12 ^{ns}	0.18 ^{ns}	0.58 ^{ns}	0.11 ^{ns}
Medium (M)	1	5.57*	0.16 ^{ns}	4.38*	5.15*
Saccharose concentration (SC)	7	31.32***	74.32***	66.1***	21.44***
Interaction (MxSC)	7	7.69**	23.16***	11.68***	8.95**

ns, *, **, ***: Non significant or significant at p≤ 0.05, 0.01 and 0.001, respectively.

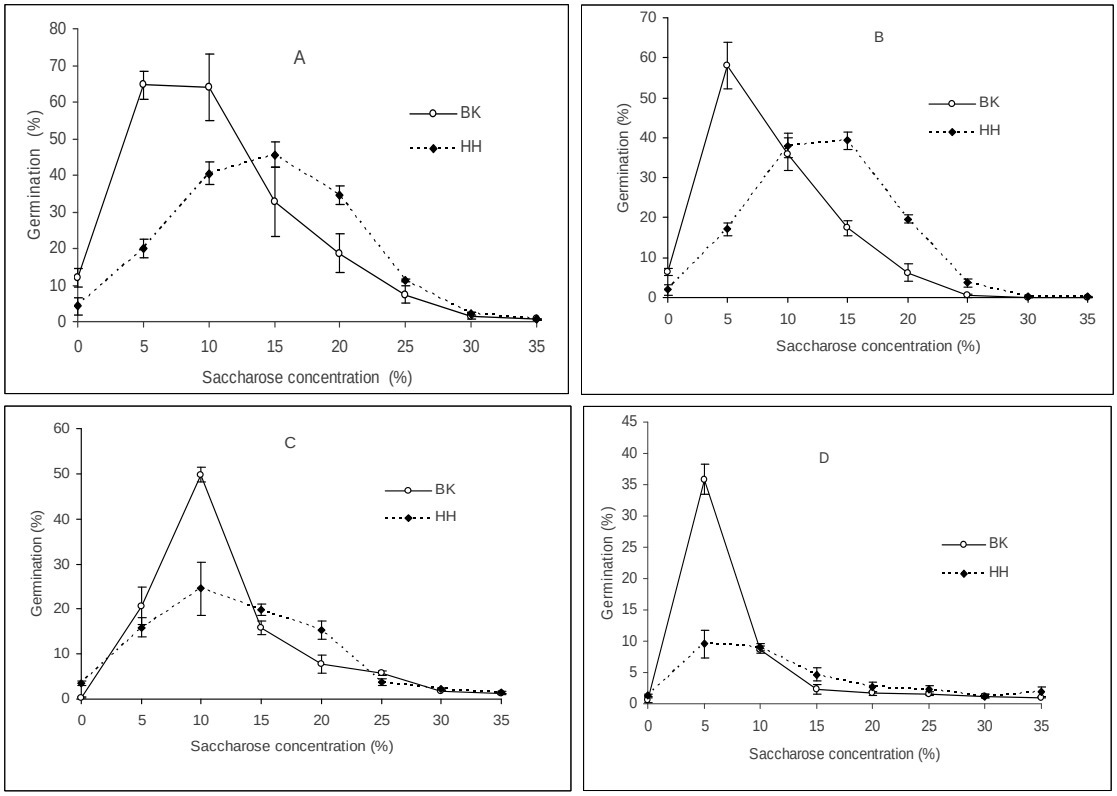

Figure 2. Effects of Brewbaker & Kwack (BK) and Heslop-Harrison (HH) base media and saccharose concentration on *in vitro* pollen grains germination of four *Cola* species: A) *C. ballayi*; B) *C. acuminata*; C) *C. anomala*; D) *C. lepidota*.

The optimum saccharose concentration in both media was 10 % and the optimum germination rates were 49.82 ± 1.53 % and 24.52 ± 5.93 % on BK and on HH respectively. Germination rate was lower in saccharose concentrations between 25 to 35 %. Statistical analysis showed higher significant differences between saccharose concentrations ($p \leq 0.001$), and base media ($p \leq 0.01$), (Table 2).

The effect of base media and saccharose concentrations on pollen grains germination of *Cola lepidota* showed that the germination rate was lower (0.57 ± 0.43 %) in absence of saccharose concentration. Higher germination rates of 35.78 ± 2.39 % and 9.52 ± 2.26 % were observed on Brewbaker and Kwack (BK) and Heslop-Harrison (HH) medium supplemented with 5 % saccharose concentration (Fig. 2D). Statistical analysis showed higher significant differences between saccharose concentrations ($p \leq 0.001$), and base media ($p \leq 0.01$) (Table 2).

Studies on the effect of incubation temperature on *Cola* pollen grains germination showed (Fig. 3) that the germination rates increased with the increase in temperature. For each *Cola* genus, the optimum was achieved at 30°C and was 64.65 ± 3.76%, 58.08 ± 5.86 %, 49.82 ± 1.53 % and 35.78 ± 2.39 % for *C. ballayi, C.*

acuminata, C. anomala, and *C. lepidota* respectively. Above 30°C, germination rate considerably decreased with each species and became zero at 45°C. The statistical analysis showed that incubation temperature significantly affected ($p \leq 0.001$) germination rates of *Cola* species (Table 3).

Effect of pH on pollen grains germination showed that pollen germinated in all pH value tested (Fig. 4). The optimum germination rate and optimum pH value varied from one species to another and was: 52.17 ± 6.74 % at pH 5.1, 62.89 ± 13.49% at pH 6, 45.26 ± 1.32 % at pH 6.3 and 30.64 ± 4.26 % at pH 5.7 for *C. ballayi*, *C. acuminata, C. anomala*, and *C. lepidota* respectively. The statistical analysis revealed that pH value significantly affected ($p \leq 0.001$) the germination rate of each *Cola* species (Table 4).

Kinetics study of pollen grains germination and pollen tube elongation of *C. ballayi* (Fig. 5), *C. acuminata* (Fig. 5) *C. anomala* (Fig. 5), and *C. lepidota* (Fig. 5) showed that 17.4 ± 2.22 %, 11.15 ± 0.54 %, 26.15 ± 2.48 % and 14.67 ± 2.25% germinated respectively after one hour. The germination rate increased with the incubation time and became stable after six or seven hours and was 51.07 ± 0.89 %, 52.36 ± 6.83 %, 44.76 ± 0.99 % and 33.07 ± 3.6 % for *C. ballayi*, *C. acuminata, C. anomala*, and *C. lepidota* respectively. Pollen tube length was lower after one hour of incubation: 60.2 ± 22.49 µm, 64.63 ± 16.25 µm, 200.06 ± 75.0µm and 100.0 ± 50.0 µm for *C. ballayi*, *C. acuminata, C. anomala*, and *C. lepidota* respectively and it increased with the incubation time and became stable after seven hours. The optimum pollen tube length was: 913.85 ± 189.51 µm, 1031.37 ± 240.71 µm, 980.53 ± 120.32 µm and 653.09 ± 195.59 µm for *C. ballayi*, *C. acuminata, C. anomala*, and *C. lepidota,* respectively.

Table 3. F values from ANOVA for the effect of temperature on *Cola* pollen grains germination

		Species			
Source of variation	df	*C. ballayi*	*C. acuminata*	*C. anomala*	*C. lepidota*
Block	2	0.14 ^{ns}	3.91*	3.3 ^{ns}	0.99 ^{ns}
Temperature (ºC)	4	57.78***	82.16***	119.03***	41.19***

ns, *, ***: Non significant or significant at $p \leq 0.05$ and 0.001, respectively.

Table 4. F values from ANOVA for the effect of pH on *Cola* pollen grains germination.

		Species			
Source of variation	df	*C. ballayi*	*C. acuminata*	*C. anomala*	*C. lepidota*
Block	2	0.89 ^{ns}	8.92 ^{ns}	1.8 ^{ns}	0.89 ^{ns}
pH	8	51.04***	34.74***	25.63***	7.63***

ns, ***: Non significant or significant at 0.001, respectively.

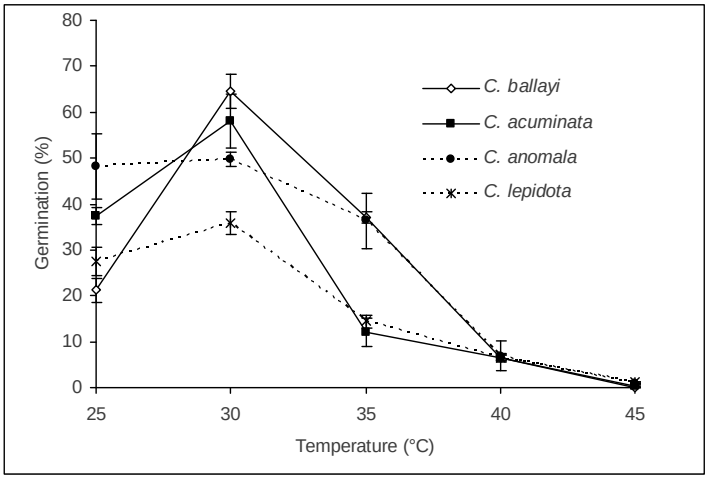

Fig. 3. Effects of incubation temperature on *in vitro* pollen grains germination of *Cola.*

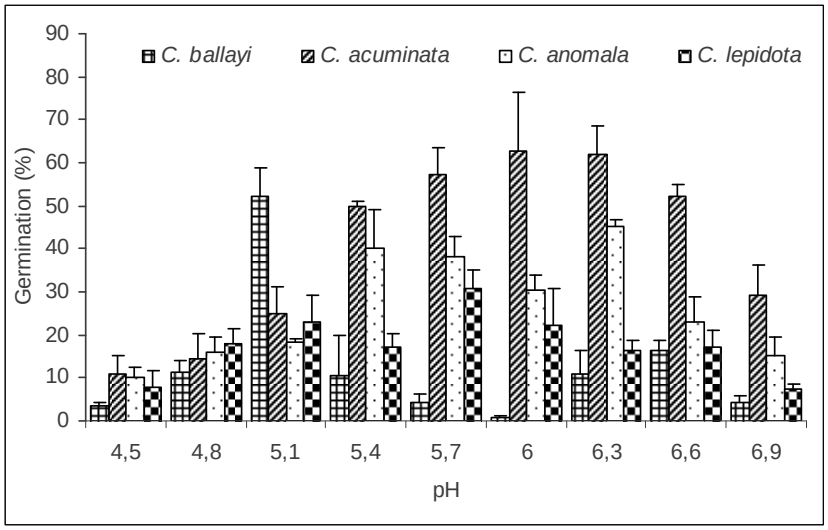

Figure 4. Effect of pH on *in vitro* germination of pollen grains of *Cola.*

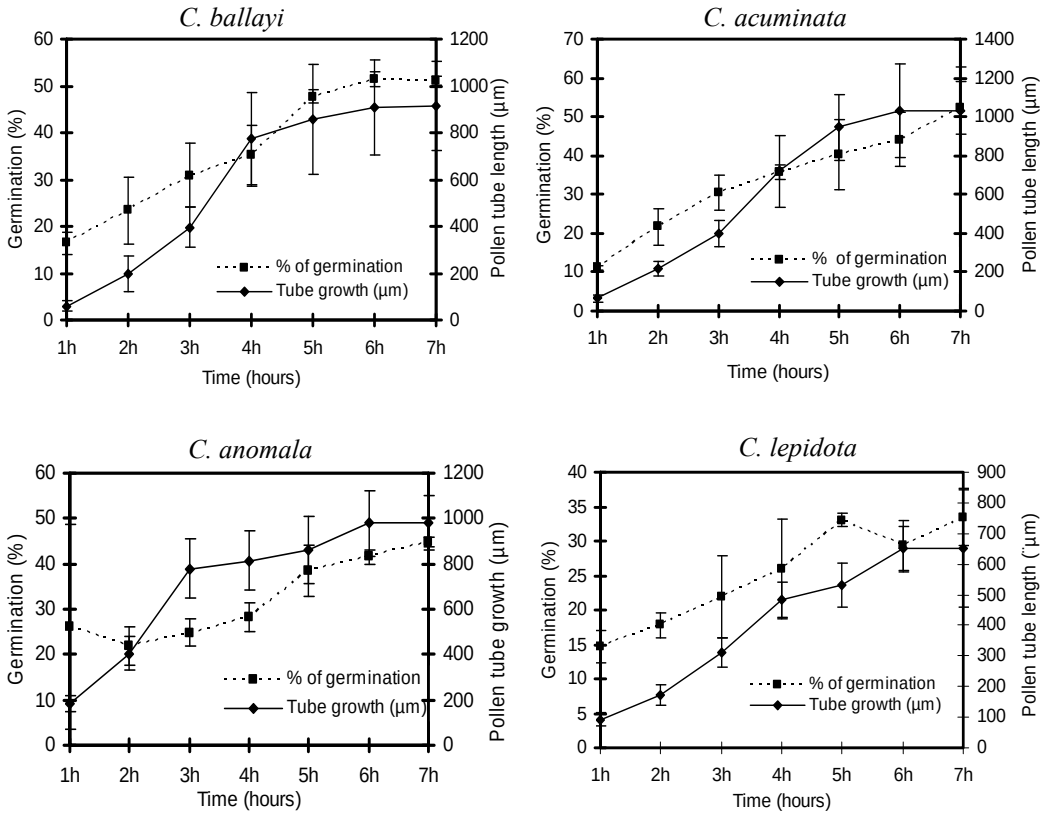

Figure 5. Germination kinetics (%) and pollen tube elongation in pollen grains of *Cola* species with time during 7 hours of incubation in the dark at 30°C.

4. Discussion and Conclusion

Morphological studies of the pollen of *C. ballayi*, *C. acuminata, C. anomala*, and *C. lepidota* showed that they were tricolporate and smooth. According to the classification of Brewbaker (1967), these pollen grains belonged to the group of bi-cellular with thick exine pollen and could undergo natural dormancy after shedding which is favourable for longer conservation (Cerceau-Larrival and Challe 1986). Pollen with many apertures have higher germination capacity than mono aperture

pollen (Youmbi, 1993).

In vitro germination capacity revealed that for each species, pollen could not germinate in absence of saccharose. This was consistent with Youmbi (1993) findings on *Catharanthus roseus* species. Saccharose plays an osmotic role in culture media (Visser 1955). In addition, it is used as source of nutrition for pollen tube growth (O'Kelly 1955; Visser 1955). The highest germination rates for the four varieties were obtained with 5 % saccharose for *C. acuminata* and *C. lepidota*, 10 % saccharose for *C. ballayi,* and *C. anomala*, after seven hours of incubation on BK medium. Rate of germination was 51.07 ± 0.89 %, 52.36 ± 6.83 %, 44.76 ± 0.99 % and 33.07 ± 3.6 % for *C. ballayi*, *C. acuminata, C. anomala*, and *C. lepidota* respectively. 10 % saccharose allowed an appropriated osmotic potential for fast pollen grains germination and pollen tube elongation of *Nicotiana tabaccum* (Loguercio, 2002). The optimum saccharose concentration varied from one species to one another belonging to the same family. Lower germination rates were obtained at higher saccharose concentration (30 and 35 %). This will be due to an alteration of pollen tube membrane affecting the infiltration of metabolic and ions into the medium. *C. lepidota* was the species with lower optimum germination rate. The low percentage of germination observed on the pollen of *C. lepidota* could be due to environmental factors such as temperature and humidity of the ecological zone where the species grows, or may be attributed to bad weather related to the transport of harvested pollen from the site to laboratory.

Effect of temperature on pollen grains germination suggested that the incubation temperature was an essential factor for pollen germination. Higher temperature values (40 and 45°C) inhibited pollen germination of the four *Cola* species. The germination was optimum at 30°C for *C. ballayi*, *C. acuminata, C. anomala*, and *C. lepidota.* These findings are similar to those reported on pollen grains germination of some tropical species: *Eucharis grandifolia*, *Euphorbia milii* and *Adenium obesum* (Youmbi, 1993).

pH value considerably affected the pollen grains germination and the optimum value varied from one species to another and were in contrast to findings of Youmbi et al. (1998). The optimum pH value of *C. acuminata* is similar to that reported on another tropical species (*Pachypodium lamerei)* pollen germination.

After 7 hours of incubation, the optimum germination rates were lower as compared to those obtained after 24 hours on BK medium supplemented with optimum saccharose concentration. Thus, the pollen tube elongation was maximal. These results are similar to those obtained on *Cola* spp. fresh pollen after 24 hours of incubation (Donfack, 2005).

In conclusion, results obtained in this study revealed that *Cola* pollen grains have good morphological characteristics which allow good germination and long term conservation. But Scanning Electron Microscope (SEM) should be investigated for exine structure and apertural description. The germination is optimum in BK medium supplemented with 5 or 10 % saccharose concentration incubated at 30°C. Pollen grains of wild species (*C. lepidota*) exhibited lower germination rate compared to cultivated species.

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