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Germination of *Cola anomala* (K. Shum.) Shott and Endl seeds: effects of provenance, substrate and dehydration

Gabriel KANMEGNE[1*], Mariette ANOUMA'A[1], FOTSO[2], Hermann Désiré MBOUOBDA[2], Delphine Ache MBIBONG[1] and Dénis Ndoumou OMOKOLO[3]

[1]*Department of Plant Biology, Faculty of Science, University of Dschang, P.O. Box 67 Dschang, Cameroon.*
[2]*Department of Biology, Higher Teacher's Training College of Bambili, University of Bamenda, P.O. Box 39 Bamenda, Cameroon.*
[3]*Department of Biological Sciences, Higher Teacher's Training College, University of Yaoundé I, P.O. Box 47 Yaoundé, Cameroon.*
[*]*Corresponding author, E-mail: gkanmegne@yahoo.fr; Tel: + (237) 677 49 00 69*

ABSTRACT

As a step in the process of *Cola anomala* domestication, investigations were undertaken on germination requirements and desiccation tolerance of its seeds. Three seed provenances (Bamenda, Bayangam and Dschang), three substrates (forest top soil, river sand and a mixture of forest top soil and river sand) and two photoperiods (12 hours/day and, continuous darkness) were investigated for their effects on seed germination. To evaluate their desiccation tolerance, fresh seeds were dried at room temperature for 16 days during which seed moisture content, seed germination percentage and electrical conductivity of seed leachate were monitored at two-day intervals. Results showed that germination percentages were significantly ($P < 0.05$) higher both on forest top soil alone (86.04 ± 4.8%), and on a mixture of forest top soil and river sand (83.56 ± 4.5%), than on river sand alone (69.96 ± 4.7%). Seeds from Bamenda showed a higher germination percentage (91.4 ± 4.7%) than those from either Bayangam (77.36 ± 4.7%) or Dschang (70.8 ± 4.8%). The desiccation tolerance test revealed that as response to drying, the mean germination percentage was first slightly reduced as moisture was lost, then declined considerably at moisture content below 50.28%. Total germination failure was observed when seed moisture reached 32.24%. Electrical conductivity of seed leachate exhibited a strong correlation with loss of viability as well as with desiccation. It is concluded that there is a provenance-related variation in *C. anomala* seed germination. The best substrate for germination is forest top soil supplemented or not with river sand in a 1/1 (v/v) ratio. *C. anomala* seeds are desiccation-sensitive and their storage behaviour is recalcitrant.

Keywords: Desiccation tolerance, critical moisture, lethal moisture, electrical conductivity, electrolyte leakage, storage behaviour.

INTRODUCTION

Cola is a tropical African genus that belongs to the Sterculiaceae family (Russel, 1955). The genus comprises about 140 species, but the most commonly used are *Cola nitida* (Vent.) Schott and Endl, *Cola acuminata* (Pal. de Beauv) Schott and Endl and *Cola anomala* (K. Schum) Shott and Endl

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(Russel, 1955; Mabberley, 2008). These three species are cultivated by subsistence farmers in association with cacao and/or coffee as shade plants, for their edible seeds commonly called kola nuts (Duke, 2001). Kola nut has for hundreds of years served as an important article of internal trade in Nigeria and other parts of Africa (Asogwa et al., 2012a). It has been an item of trade in West Africa and in the Trans-Saharan trade routes for many centuries (Gebissa, 2006). The spread of kola nuts has resulted from its reputation as a stimulant, increasing energy and strength, dispelling drowsiness and staving off hunger. These properties have been attributed to the large amounts of caffeine and smaller amounts of the obromine, kolatin and glucose it contains, all of which act as stimulants and may be mildly addictive (Blades, 2000; Niemenak et al., 2008). It is also used to flavor drinks and in the manufacture of pharmaceuticals, and it is exported to Europe and North America for these purposes (Burkill, 2000). The most recent and remarkable advancement in kola by-product utilization is the use of kola pod husks in the replacement of up to 60% of the maize used in the formulation of poultry feed (Hamzat and Babatunde, 2001). Kola nut is one of the many non-timber forest products that are of socio-economic importance. Its commercialization in both the domestic and national markets raises the standard of living of those involved in its trading activities (Gebissa, 2006; Asogwa et al., 2012b).

Given the socio-economic importance and the possibility of growing these trees in farms, *Cola* sp (*C. anomala, C. acuminata* and *C. nitida*) have been suggested as candidates for domestication by agro forestry. As an important step in the process of domestication of these species which constitute a vital resource for millions of people in sub-Saharan Africa, research on seed germination physiology is needed. There have been some attempts to domesticate *Cola* species, but this concerned only *C. nitida* and *C. acuminata*. For these two species, seed handlings, plant regeneration through seeds, and vegetative propagation have been widely studied (Famaye et al., 2007; Mbete et al., 2011). However, for *C. anomala* very little has been studied on the process of plant regeneration, and the characteristics of seed germination have not been documented to date. Moreover, classification of seed storage behaviour has not been made for this last species. Indeed, among the different species of *Cola* producing edible nuts, *Cola anomala* has suffered neglect in the areas of research and development despite its socio-economic importance.

As an important step in the process of *Cola anomala* domestication, this work was undertaken to determine: 1) the influence of three important factors (provenance, photoperiod and substrate) on the germination of seeds, and 2) the desiccation sensitivity and storage behaviour classification of seeds.

MATERIALS AND METHODS

Seed material

Seeds used in the present study originated from three localities situated in the western highlands of Cameroon: Bayangam (5°18’N, 10°29’E, altitude 1500 m), Bamenda (5°47’N, 10°10’E, altitude 1300 m) and Dschang (5°27’N, 10°3’E, altitude 1400 m). In each locality, mature and disease-free pods were harvested from a minimum of 10 randomly selected trees in March 2012. A minimum of 100 pods (containing 1 to 10 seeds each) were harvested from each site and immediately brought to the Laboratory of Applied Botany, Department of Plant Biology, University of Dschang. In the laboratory, seeds were extracted from pods 24 hours after harvest and processed by removing their white and soft testa to obtain red or pink coloured fresh nuts, which were used for further experiments.

Germination assay

Seeds from each provenance were sown in black plastic perforated polythene bags (20 cm high and 10 cm diameter) filled with substrate which was either forest top soil, river sand or a mixture of forest top soil and river sand in 1:1 (v/v) ratio. As light regime (photoperiod) effect was one of the topics of investigation, sowing was done in such a way that ¾ of the volume of seed was buried in the substrate while the remaining ¼ was visible above. The seeded polythene bags were then placed in the nursery (22 ± 3 °C) at two different photoperiods: natural photoperiod (12 hours/day) and continuous darkness. Darkness was provided by placing the seeded polythene bags in wooden boxes (1m x 1m x 0.5 m) whose internal walls were lined with black and thick polythene paper.

To investigate on *Cola anomala* seed germination, a total of 1350 seeds were sown in three blocks of a split-split plot experimental design. Each main plot contained three substrates (Forest Top soil, River Sand and 1:1 (v/v) mixture of Forest Top soil and River Sand), whereas three different provenances (Bamenda, Bayangam and Dschang) were tested at the subplot level. At the sub-subplot level, two photoperiods (12 hours/day and continuous darkness) were investigated. At each level, treatments were assigned at random to experimental units so as to have 3 substrates X 3 provenances X 2 photoperiods X 3 replications X 25 seeds. Manually, water was applied daily to the seeded polythene bags using a sprayer, so that the medium (substrate) was kept moist without getting waterlogged. Nuts with radicle protrusions and/or with the emergence of the plumule were recorded as having germinated. The percentage of germination was recorded 20 days after seeding.

Desiccation tolerance test

Mature seeds harvested from a single tree in Bamenda were used for the desiccation tolerance test. Fresh seeds were extracted from mature pods 24 hours after harvest and processed as described above. They were then spread in a single layer on the laboratory bench top and left to dry under shade at laboratory temperature (25 ± 2 °C). Laboratory relative humidity was 55 ± 5%. Seed samples were withdrawn every 2 days for moisture content measurement, conductivity test and germination test.

Moisture content (MC%) was determined by the oven dry method which consisted in weighing seeds before and after drying them in the oven at 103 °C for 17 h. Moisture content, which was expressed as a percentage of fresh weight, was calculated using the following formula:

$$MC\% = [(FW - DW) \div FW] \times 100.$$

Where FW (fresh weight) is weight of sample before drying and DW (dry weight) is weight of sample after drying (ISTA, 2004). The value of the moisture content for each drying time was the mean of six measurements (six replications of one seed each).

For the conductivity test, a square of seed piece (cotyledon) was cut, weighed and soaked in distilled water in a 100 ml beaker (i.e., 3 g of seed piece in 50 ml of distilled water). The beaker was covered and left in the laboratory at 25 °C for 24 h. Two beakers with 50 ml of distilled water, but no seed piece, were treated similarly as blanks to determine the base conductivity of the water. After 24 h, the seed piece was strained from the water and conductivity of the water was measured using a HACH Model of Conductivity/TDS Meter. The mean conductivity of the blanks was subtracted from each sample reading. Conductivities were expressed in microSiemens (µS). Six replications were carried out at each drying time.

At 2-day intervals, three replications of 25 seeds were withdrawn for the germination test which was carried out in the nursery at 22 ± 3 °C, natural photoperiod (i. e. 12 hours of light per day) and in black polythene bags

filled with forest top soil as substrate. The seeded polythene bags were then regularly watered and germination percentages were recorded for 20 days as described above.

Statistical analysis

Data analyses were performed using SPSS 12.0 software package. The dependent variable was the mean germination percentage, whose data were transformed into arcsine square root values before statistical analysis. Analyses of variance (ANOVA) were performed to detect the level of significance of the main effects of parameters such as seed provenance, germination substrate and photoperiod, as well as the significance of their interaction effects. Means that exhibited significant differences ($P < 0.05$) were further compared using Duncan's multiple comparison test or t-test as appropriate.

The 2-tailed Pearson correlation coefficient was used to establish correlations between the different seed variables investigated (moisture content, germination percentage, and electrical conductivity of leachate). The critical water content of seeds was recorded at the point in which the germination significantly decreased while the lethal water content was recorded at the point where there was total failure of germination.

RESULTS

Analysis of variance (Table 1) showed that the germination percentage was significantly ($P = 0.03$) influenced by substrate and highly significantly ($P = 0.003$) influenced by the provenance. As shown in Table 2, germination percentages were significantly ($P< 0.05$) higher both on forest top soil (86.04 ± 4.8%) and on forest top soil + river sand mixture (83.56 ± 4.5%), than on river sand alone (69.96 ± 4.7%). Seeds from Bamenda showed a higher germination percentage (91.4 ± 4.7%) than those from either Bayangam (77.36 ± 4.7%) or Dschang (70.8 ± 4.8%). There was no significant difference between the germination percentages of seeds from Bayangam and Dschang. The highest germination percentage (98.22 ± 8.2%) was recorded with seeds from Bamenda sown on forest top soil, while the lowest (65.2 ± 8.4%) was recorded with seeds from Dschang sown on river sand.

Results of the desiccation tolerance test showed that fresh seeds of *C. anomala* with initial moisture content of 67.57 ± 0.89% had a germination percentage of 90 ± 1.15% and the conductivity of leachate was 30.43 ± 1.33 µS. When seeds were dried, the germination percentage declined gradually with decreasing moisture content, while the electrical conductivity of leachate increased (Figure 1).

From 0 to 10 days of drying time, the moisture content decreased from 67.57 ± 0.89% to 50.28 ± 2.53% and the germination percentage was maintained between 90% and 55.33%, meanwhile the electrical conductivity of leachate was maintained between 30.43 µS and 53.13 µS. At moisture content below 50.28%, the germination percentage drastically dropped to 12 ± 1.15% at 12 days of drying time. At the same time, the electrical conductivity of leachate abruptly increased to 178.5 ± 17.7 µS. The value of 50.28% was then recorded to be the critical moisture content. After 16 days of drying, the moisture content had reduced to 32.24 ± 1.08% and germination was 0%. The value of 32.24% was consequently recorded as the lethal moisture content. Seed leachate conductivity was highly and negatively correlated both to the germination percentage ($r = – 0.93$) and to moisture content ($r = – 0.91$), meanwhile the germination percentage and moisture content were positively correlated ($r = 0.98$) (Table 3).

Table 1: ANOVA of the effects of substrate, photoperiod, seeds provenance and their interactions on mean germination percentage of *C. anomala* seeds at 20 days after sowing.

Effect	DF	*F*-value	*P*-value
S	2	3.39	0.03
Ph	1	3.29	0.07
Pr	2	5.94	0.003
S x Ph	2	0.64	0.52
S x Pr	4	0.32	0.85
Ph x Pr	2	0.69	0.5
S x Ph x Pr	4	0.1	0.98

Notes: DF, Degree of freedom; S, substrate; Ph, photoperiod; Pr, provenance; S x Ph, substrate x photoperiod interaction; S x Pr, substrate x provenance interaction; Ph x Pr, photoperiod x provenance interaction; S x Ph x Pr, substrate x photoperiod x provenance interaction

Table 2: Germination percentages of *C. anomala* seeds from different provenances 20 days after sowing on different germination substrates.

	Substrate			
Provenance	Top soil	Top soil + River sand	River sand	Provenances' main effect
Bamenda	98.22 ± 8.2[a]	98.0 ± 8[a]	78.0 ± 8.2[a]	91.4 ± 4.7[a]
Bayangam	82.4 ± 8.2[b]	83.0 ± 8.2[b]	66.7 ± 8[b]	77.3 ± 4.7[b]
Dschang	77.5 ± 8.4[b]	69.7 ± 8.2[b]	65.2 ± 8.4[b]	70.8 ± 4.8[b]
Substrates' main effect	86.04 ± 4.8	83.56 ± 4.5	69.96 ± 4.7	
	ns			
	*			

Notes: Within the same column, means ± SEM followed by the same letter(s) are not significantly different at the 5% level according to Duncan multiple comparison test. ns, no significant difference between Top soil and Top soil+River sand mixture; *Significant difference at 5% level between River sand and the two other substrates.

Table 3: Two tailed Pearson correlation coefficients between different *Cola anomala*'s seeds parameters.

	Moisture content	Conductivity	Germ. percentage
Moisture content	1	-0.915**	0.983**
Conductivity		1	– 0.937**
Germ. percentage			1

**, correlation is significant at p≤ 0.01.

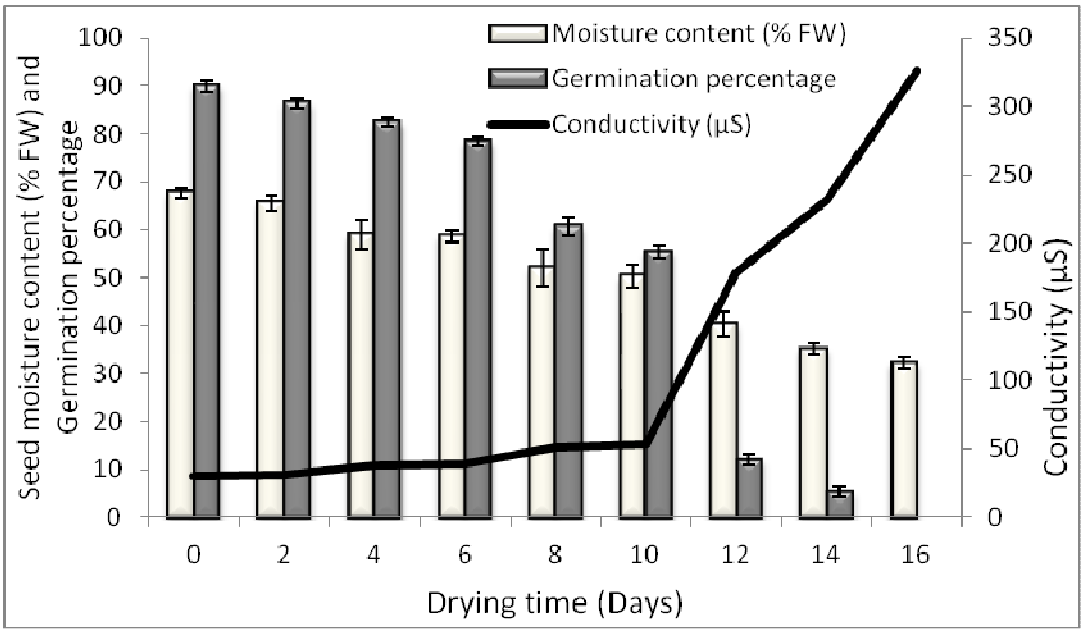

Figure 1: Variation in moisture content, germination percentage and leachate conductivity of *Cola anomala* seeds during drying.

DISCUSSION

Seed provenances have displayed significant differences in germination percentages of *C. anomala* seeds. Some authors (Loha et al., 2006) showed a relatively low correlation between seed provenances and their germination parameters. In most plant species nevertheless, seeds vary in their degree of germinability between and within populations and between and within individuals (Bischoff et al., 2006; Kanmegne and Omokolo, 2008). Some of this variation has been suggested to be of genetic origin (Bischoff et al., 2006), but much of it is known to be phenotypic, caused by the local conditions under which the seed matured (Cedan et al., 2013). Indeed, germination characteristics are not only affected by the current environmental conditions but also by conditions experienced by the mother plants in the previous generation (Khurana and Singh, 2001; Fenner and Thompson, 2005). Nevertheless, it cannot be concluded from the present study which factor (topographic or climatic characteristics of locations or genetic characteristics of mother trees) caused the provenance-related variation in *Cola anomala* seeds germination.

One of the most significant factors for seed germination in the nursery is the type of substrate. A substrate needs to have adequate aeration and moisture for germinating seeds (Khurana and Singh, 2001). The results of the present study showed that forest top soil and a mixture of forest top soil and river sand showed a higher germination percentage compared to river sand alone. As seeds were only partially buried, there was no problem of seed aeration. The differences among different substrates for their germination percentage as reported here may be attributed to the differences in moisture retention. It is well known that both forest top soil and the mixture of forest top soil and river sand have higher moisture retention capacity than river sand alone. Germination of *C. anomala* seeds

may have been enhanced by the constantly moist condition, which is not possible in sand. Similar results have been reported for seeds of many others species (Benvenuti, 2003; Rodriguez et al., 2014), although ISTA (2004) has suggested sand as a suitable medium for seed germination in some species.

In relation to the desiccation sensitivity test, the moisture content value of 67.57% recorded for fully matured fresh seeds of *Cola anomala* was very high. According to MSBP (2002), such moisture content indicates that seeds are potentially immature or are in the post abscission phase. This is however applicable to orthodox seeds which go through the process of maturation drying. Recalcitrant seeds on the contrary do not undergo maturation drying and even after shedding from the mother plant they have high moisture content ranging from 30 to 70%, leading to their wetness (Martins et al., 2003; MSBP, 2005).

The capability of seeds to germinate was greatly affected by moisture content. Seeds with high initial moisture content (67.57%) showed a maximum germination percentage (90%). The mean germination percentage then declined as the moisture content decreased, and below 32.24% moisture content there was no seed germination. The loss in mean germination percentage as a result of seed dehydration indicated that *C. anomala* seeds can be categorized as desiccation-sensitive. This was not surprising, since *C. anomala* seeds exhibit most of the characteristics of recalcitrance such as being fleshy, large in size and produced from plants growing in the humid forest environment (Thomsen, 2000). When fresh recalcitrant seeds begin to dry, their viability is first slightly reduced as moisture is lost, but begins to decline considerably at a certain moisture level termed the critical moisture content or lowest safe moisture content (Walters et al., 2001; Wesley-Smith et al., 2001). The value of 50.28% recorded in the present study as the critical moisture content is higher than those reported for many other recalcitrant tree seeds such as *Garcinia kola* seeds (Agyili et al., 2007; Asomaning et al., 2011) and *Corypha umbraculifera* seeds (Viji et al., 2013), but similar to values which have been reported for *Archontophoenix alexandrae* (Martins et al., 2003) and *Euterpe edulis* (Panza et al., 2007) seeds. This is an indication that the critical moisture content of recalcitrant seeds varies greatly among species.

Electrical conductivity of seed leachate exhibited a strong correlation with loss of viability as well as with desiccation. This indicates that measurement of leachate conductivity can be considered as a valuable tool for viability test in *C. anomala* seeds, as previously reported for many others species as *Garcinia kola* (Asomaning et al., 2011) and *Vicia sativa* (Samarah, 2006). Electrolyte leakage increased abruptly as seeds were dried below the critical moisture level and reached its maximum value at the lethal moisture level, corroborating results obtained from seeds of *Euterpe edulis* (Panza et al., 2007) and *Syzygium cuminii* (Abbas et al., 2003).

It has been suggested that there is a linear relationship between seed desiccation and membrane damage, while electrical conductivity and membrane damage are also directly correlated events (Khan et al., 2003). Indeed, electrolyte leakage from tissues can be used to indicate the effectiveness of membranes as barriers to solute diffusion. While relatively low levels of leakage indicate that cellular membranes are semi permeable, high levels of leakage indicate damage to membranes (Sacandé et al., 2001). Many studies provide evidence that if dehydration stress disrupts membrane integrity in desiccation sensitive seeds, then increases in

the amount of solutes leaked may be detectable in response to dehydration, and these increases should be associated with loss of viability (Walters et al., 2001; Wesley-Smith et al., 2001). The results presented here are complementary to these findings and clearly indicates that cellular membranes of desiccation-sensitive *Cola anomala* seeds may have been damaged as seeds were dried further as observed in the increases in the levels of solute leakages. Excessive dehydration of the seeds beyond the critical moisture content may have severely disrupted the integrity of the cellular membranes of seed tissues, resulting in the uncontrollable rate of solute losses from seeds. This is consistent with the concepts that membranes in desiccation-sensitive seeds are damaged by dehydration below the critical moisture content and are unable to reform completely during imbibitions (Kernode and Finch-Savage, 2002; Berjak et al., 2007).

Conclusion

The *C. anomala* seeds are clearly desiccation-sensitive, with 50.28% and 32.24% as values of the critical and the lethal moisture contents respectively. Loss in seed viability during dehydration was found to be associated with increased electrolyte leakage, following the disruption of cellular membranes integrity. For propagating *C. anomala* from seeds, it is recommended that fresh seeds from mature pods be sown without being previously dried, and that seeding be done in substrate composed of either forest top soil or a mixture of forest top soil and river sand in a 1:1 (v/v) ratio.

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