IN VITRO SEEDS GERMINATION AND SEEDLING GROWTH OF CASHEW (*ANACARDIUM OCCIDENTALE* L.)

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ABSTRACT

In Côte d'Ivoire, the yield of cashew nuts is low. This situation has led to the use of improved clonal-based material for the expansion or establishment of new plantations. To obtain a mass propagation of such elite materials, application of tissue culture becomes very important. However, it is difficult to obtain viable explants derived from mature plants developing in field because of high concentrations of disinfectants required for surface sterilization. Explants excised from *in vitro* seedlings are therefore the most suitable for the micropropagation of cashew. The objective of this study was to identify the type of seeds suitable for *in vitro* germination and plantlets development. Seeds without shell with entire cotyledons (SWSEC), seeds without shells with half cotyledons (SWSHC) and the embryonic axis (EA) were placed on the basal medium of Murashige and Skoog (MS) without any plant growth regulators. The final rate of germination ranged from 75 to 95 %, the time to have 50 % germination and the average germination time were statistically identical with the three types of seeds. Higher plants (7cm) with larger numbers of leaves, which are more vigorous were obtained with SWSHC. This type of seeds is therefore more suitable provide seedling used as explant sources for the micropropagation of the cashew tree.

Key words : *Anacardium occidentale* ; germination ; micropropagation

RESUME

GERMINATION* IN VITRO *DES GRAINES ET CROISSANCE DES VITROPLANTS DE L'ANACARDIER (ANACARDIUM OCCIDENTALE L.)

En côte d'Ivoire, le rendement en noix de l'anacardier est faible. Cette situation a conduit à l'utilisation de matériels améliorés pour l'extension ou la mise en place de nouvelles parcelles. Pour réaliser la propagation en masse de tels matériels, les méthodes in vitro *sont indispensables. Cependant, il est difficile d'obtenir des explants viables prélevés sur des plantes au champ après leur désinfection. Les explants provenant des vitroplants sont donc les plus adaptés à la micropropagation de l'anacardier. Cette étude vise à identifier le type de graine approprié pour la germination* in vitro *et la croissance des vitroplants chez l'anacardier. Les graines sans coque avec les cotylédons entiers (GSCCE), les graines sans coque avec demi cotylédons (GSCDC) et l'axe embryonnaire (AE) ont été placés sur le milieu Murashige et Skoog (MS). Les taux finaux de germination ont varié de 75 à 95 %, le temps pour avoir 50 % de germination et le temps moyen de germination ont été statistiquement identiques pour les trois types de semences. Les vitroplants de plus grande taille (7cm) avec un plus grand nombre de feuilles (5 feuilles) ont été obtenus à partir de GSCDC. GSCDC est donc recommandé pour l'obtention de source d'explants pour la micropropagation de l'anacardier.*

Mots clés : Anacardium occidentale *(L) ; germination ; axe embryonnaire ; micropropagation*

INTRODUCTION

Cashew nut (*Anacardium occidentale* L.), an important edible nut crop, is cultivated in tropical areas of India, Brazil, and Africa (Wickramasinghe, 2002). Cashew nut and liquid from nut shell (CNSL) are the two most important parts which have used in numerous medical and industrial applications (Rajesh *et al*., 2009). Cashew kernel is a rich source of protein, carbohydrate, unsaturated fats and minerals, which helps to avert anemia, and nervous disorders. Cashew contained 47 % of fat with a balanced nutritive profile and vitamins. Health benefits of cashews come next to almonds with additional high unsaturated fats (Thanishka, 2009).

In Côte d'Ivoire cashew nut has quickly attracted interest so that the country has become since 2015, the world's largest producer before India with 702 000 tonnes of cashew (MINAGRI, 2016). Despite the importance of production, the yields of walnuts in the Ivorian orchards remain low, of the order of 350 to 500 kg/ha, because of plantations created with unimproved plant material and unsuitable peasant farming practices (Djaha *et al.,* 2012). This is mainly due to the establishment of plantation with seedlings of no descripted origin. Existing area with cashew plantation of such material needs to be replanted with clonal material of elites lines to boost the production and reduce the import of raw nuts from other countries. This programme requires planting materials in large number. Currently, cashew is vegetatively propagated by softwood grafting. *In vitro* propagation technique in cashew needs to be developed to supplement this conventional method (Thimmappaiah *et al*., 1999).

Some degree of progress with the application of micropropagation in cashew has been achieved. There have been many reports on micropropagation in cashew via organogenesis (Lievens *et al*., 1989 ; Leva and Falcone, 1990 ; Mantell *et al*., 1998). Such investigations were carried out with microcuttings and young leaves from seedlings, cotyledons from mature seed, nodal cuttings, shoot tips, and cotyledonary nodes from *in vitro*-raised seedlings.

In cashew, despite the progress, *in vitro* culture encounters several problems, including the viability of explants after surface sterilization. Silva *et al* (2011) and Das and Jha (1996) noted that the explants collected in the field survived with difficulty because of the high level of disinfectant required for the decontamination of this material.

The authors initially recorded 3 % and 25 % survival for shoot tips and nodal explants of field-grown twigs, respectively, subjected to thorough sterilization. Thereafter, few explants that survived sterilization turned brown and died after 20 days of culture. Kembo and Hornung (1999) also reported that, the rate of survival of the treated plantlets *ex vitro* never exceeded 28 %. Thanishka (2009) reported that often, micro propagation from mature tree explants is affected by excessive contamination, phenolic exudation, slow growth, difficulty in elongation and rooting of micro shoots.

Therefore ; explants excised from seedlings derived *in vitro* seeds germination will be most suitable for micropropagation of elite cashew.

In vitro germination of cashew nut were reported by many authors. Ananthakrishnan *et al.* (2002) obtained *in vitro* germination of mature nuts by culturing proximal and distal part on MS medium supplemented with BAP. Seeds without shells with half cotyledons was used for germination of cashew on MS (Keshavachandran, 2004) and MS, WPM and B5 media (Thimmappaiah *et al*, 2001). However, the germination capacity of these different seeds has never been reported.

This study is aimed to establish optimal *in vitro* conditions for cashew seeds germination and seedling growth based on the responses of the seeds types used as explants. The results from this study are prerequisites to develop plant regeneration of different cashew nut landraces.

MATERIALS AND METHODS

SEED SOURCES AND SURFACE STERILIZATION

Cashew mature seeds were collected in holder plantation of Gohitafla in west center of Côte d'Ivoire (transitional woodland savannah with blocks of semi-deciduous forests). The seeds were surface sterilized sequentially during 1 min in 70 % (v/v) ethyl alcohol and 30 min in 7 % (w/v) calcium hypochlorite solution. Seeds were then rinsed and immersed in sterile distilled water for 72 hours. After imbibition, the seeds

were rinsed four times and surface sterilized a second time during 1min in 70 % (v/v) ethyl alcohol, 15 min in 7 % (w/v) calcium hypochlorite solution and rinsed abundantly with sterile distilled water. After double sterilization, seed coats were removed and three types of seeds were used for *in vitro* germination : (i) decoated seeds with cotyledon (Figure 1A), (ii) decoated seeds with half cotyledon (Figure 1B), and (iii) the embryonic axis carefully isolated after the cotyledons have been separated (Figure 1C).

Culture Media

The seeds were cultured on MS medium (Murashige and Skoog, 1962) containing. 3 % sucrose and 0.2 % activated charcoal. The pH of the culture medium was adjusted to 5.8 before adding 0.8 % (w/v) agar.

Culture Conditions

The culture medium was autoclaved at 121°C for 20 min. For all the experiments described above, the cultures on glass jar were incubated under 50 mol/m^2/s light provided by cool white fluorescent lamps for a photoperiod of 12 h at 25°C ± 2°C.

Germination and Growth Parameters

In this work, germination was defined as the appearance of a 2 mm radicle. Germination was monitored daily for each seed type until no further germination was recorded and the mean germination time (MGT) was calculated using the formula reported by Ellis and Roberts (1981)

$$MGT = \frac{\Sigma(nT)}{\Sigma n}$$

n is the number of seeds newly germinated at time T ; T represents time where seeds germinated were recorded ; Σn is the total number of seeds germinated.

The total number of the germinated seeds for each seeds type were summed up to determine the cumulative germination and the rate of germination was calculated following the procedure of Czabator (1962).

After 16 days of culture, seedlings length, and the number of leaves were evaluated.

Data analysis

The experiment was carried out in a completely randomized design with ten replicates per treatment repeated three times. Data were submitted to analysis of variance (ANOVA) to detect significant differences between means of each seed type. Means differing significantly were compared using Newman-Keuls multiple range test at the 5 % probability level using statistical software program Statistica version 7.1.

RESULTS

EFFECT OF SEEDS TYPE ON *IN VITRO* GERMINATION

On MS medium, seeds became swollen quickly and germinated after three days of culture (Figure 1). Figure 2 presents germination and contamination rate of the three seed types after 16 days of culture. The germination and contamination rate were not influenced by the different seeds type tested. Therefore, in cashew, presence of cotyledon is not a pre-requisite for germination. But the lowest contamination rate was obtained with embryonic axis.

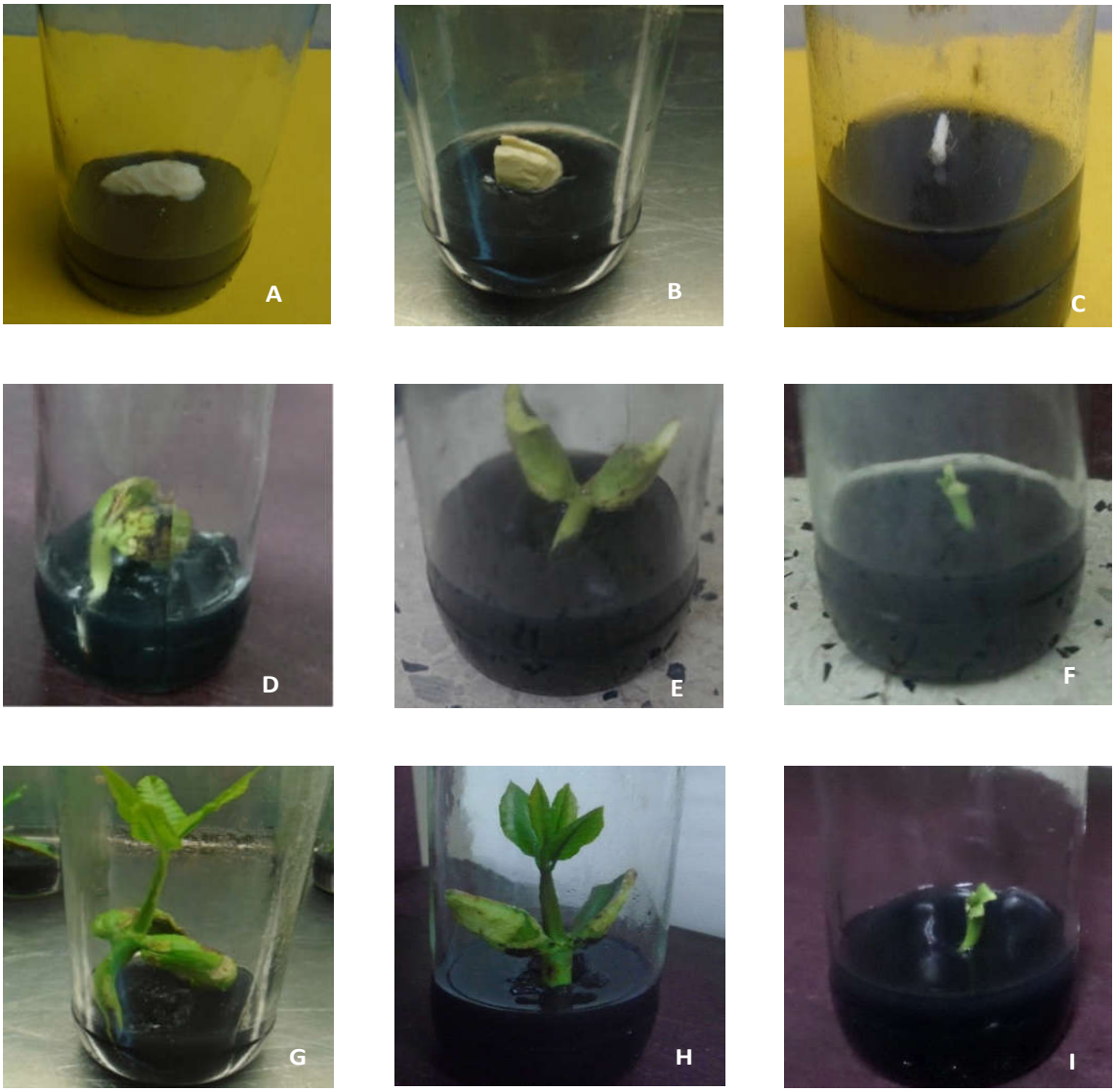

Figure 1 : *In vitro* seeds germination and seedling development of *Cashew nut*. (A, B and C) Seeds type explants on MS medium. (D, E and F) Seeds type after six days of culture on MS medium. (G, H, and I) Developed seedling derived from seeds with entire cotyledon (G) ; seeds with half cotyledon (H) ; and embryonic axis (I) after 16 days of culture on MS medium.

Germination in vitro *et croissance des vitroplants d'anacardier. (A, B et C) Types de graines sur milieu MS. (D, E et F) Types de graines après six jours de culture sur milieu MS. (G, H, et I) Croissance des vitroplants issus de graines avec cotylédon entier (G) ; graines avec cotyledon réduit de moitié (H) et axe embryonnaire (I) après 16 jours de culture sur milieu MS.*

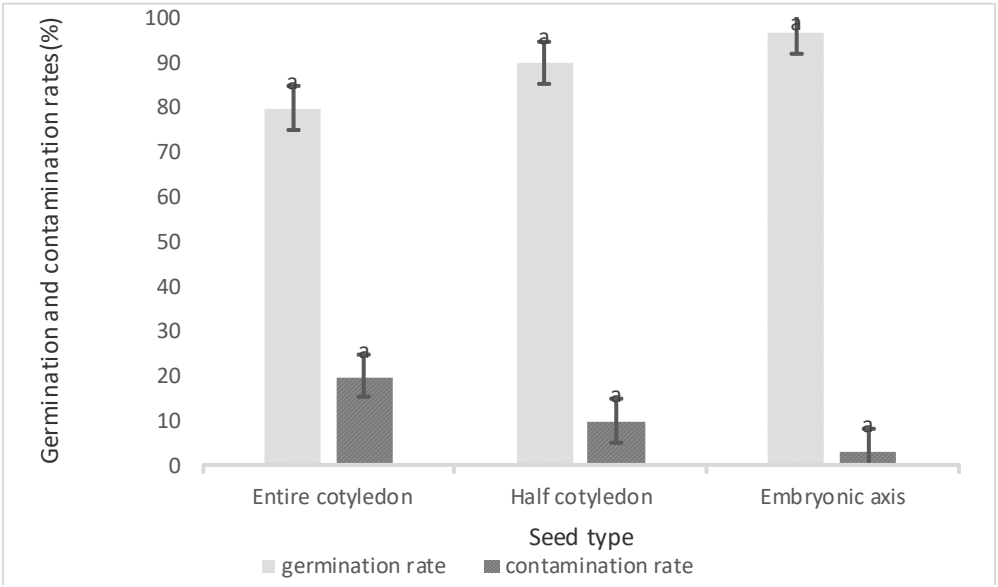

Figure 2 : germination and contaminaton rates of differents seeds types of cashew after sixteen days of culture.

Pourcentage de germination et de contamination de différents types de semence d'anacardier après 16 jours de culture.

The means germination time (MGT) of the three seeds types after two weeks of culture are presented in Table 1. The MGT and the time to obtain 50 % of germination (T50 %) were not influenced by the seeds type tested. Four days is the time required for the three seeds type germination (embryonic axis, the seeds with entire cotyledon and seeds with half cotyledon).

Table 1 : Time to obtain 50 % of germination and mean germination time of different seeds type of cashew after sixteen days of culture.

Temps pour avoir 50 % de germination et temps moyen de germination de différents types de grains d'anacardier après 16 jours de culture.

Explant type	T 50 %	Mean germination time (days)
Entire cotyledon	4.14 ± 0.59 [a]	4.20 ± 0.13 [a]
Half cotyledon	4.25 ± 0.26 [a]	4.27 ± 0.17 [a]
Embryonic axis	4.19 ± 0.14 [a]	4.23 ± 0.14 [a]
P	0.809676	0.954351

Mean in a column followed by a common letter are note significantly different at 5 % level (Newman-Keuls test) (average ± standard error).

Dans une même colonne, les chiffres suivis de la même lettre ne sont pas significativement différents au seuil α *= 5 % (test Newman-keuls) ; (± erreur standard).*

EFFECT OF EXPLANT TYPE ON OBENTION OF NORMAL PLANT

The mean values of normal plant parameters of plantlets obtained from the three explants types after 16 days of culture are presented in Table 2. Plant with well-developed root and shoots were considered as normal plant. The mean time to obtain was not influenced by the different normal plant seeds type tested. Five and six days are the times required to obtain normal plant with the three types of seeds (table 2).

Significant difference in normal plant rate was noted between explants type. The highest normal plant rate (88 %) was observed with embryonic axis followed by seeds with half cotyledon (58 %) and seeds with entire cotyledon (52 %)

Table 2 : Normal plante rate and mean time to obtain normal plant with different seeds type of cashew after sixteen days of culture.

Taux de plant normal et temps moyen d'obtention de plant normal d'anacardier après 16 jours de culture.

Explant type	Normal plante rate (%)	Mean time (days) to obtain normal plant
Entire cotyledon	52 ± 9.26 [b]	5.6 ± 1.67 [a]
Half Cotyledon	58 ± 9.20 [b]	6.2 ± 1.58 [a]
Embryonic axis	88 ± 3.33 [a]	6.4 ± 1.24 [a]
P	0.000183	2.271

Mean in a column followed by a common letter are note significantly different at 5 % level (Newman-Keuls test) (average ± standard error).

Dans une même colonne, les chiffres suivis de la même lettre ne sont pas significativement différents au seuil α *= 5 % (test Newman-keuls) ; (± erreur standard).*

EFFECT OF EXPLANT TYPE ON SEEDLINGS GROWTH

The mean values of growth parameters of plantlets obtained from the three explants types after 16 days of culture are presented in Table 3. Significant differences in performance/ development were observed among plantlets obtained with embryonic axis explants and those from, the seeds with entire cotyledon and seed type with half cotyledon. The lowest growth performances were observed with the plants derived from embryonic axis explants. The highest plantlets length (7.12 cm) was observed with plantlets obtained from the seeds type with half cotyledon followed by the plants derived from seeds with entire cotyledon (5.32 cm).

Table 3 : Plantlets length and number of leaves of cashew plantlets derived from three explants type after 16 days of in vitro culture.

Longueur des plants et nombre de feuilles de plants d'anacardier obtenus à partir de trois types d'explants 16 jours après la mise en culture.

Explant type	Plantlets length	Number of leaves
Cotyledon entire	5.32 ± 0.60 [b]	4.33 ± 0.16 [b]
Half cotyledon	7.12 ± 0.38 [a]	5.06 ± 0.10 [a]
Embryonic axis	1.27 ± 0.03 [c]	0.00 ± 0.00 [c]
P	0	0

Mean in a column followed by a common letter are note significantly different at 5 % level (Newman-Keuls test) (average ± standard error).

Dans une même colonne, les chiffres suivis de la même lettre ne sont pas significativement différents au seuil α *= 5 % (test Newman-keuls) ; ± erreur standard.*

DISCUSSION

The germination and contamination rate were not affected by the different seeds type tested. Therefore, *in vitro* germination can occur in cashew without the necessary presence of cotyledonary structure. On the other hand, continuous supply of water is needed to start and complete germination (Adu-Dapaah, 2004). The lowest contamination rate was obtained with embryonic axis. The absence of cotyledon could reduce the contamination.

Four days is the time required for the three seeds type to perform (embryonic axis, the seeds with entire cotyledon and seeds with half cotyledon). The absence of coats could explain the same time required to observe germination with the different seeds types.

The highest normal plant rate was observed with embryonic axis contrary to the seeds with entire cotyledon and seeds with half cotyledon. The source of energy that might be produced by cotyledons for embryonic axis growth is provided with the sugar contained in medium culture. The

low rate of normal plants obtained after germination of seeds with cotyledons could be explained by the health status of the cotyledons. In fact, cotyledons can be infected by pathogens either during harvesting or during seeds storage. During or after germination, infected cotyledon pathogens could reach the embryonic. This can lead to a non-development of the embryo.

The lowest growth performances were observed with the plants derived from embryonic axis explants. The highest plantlets length was observed with plantlets obtained from the seeds type with half cotyledon followed by the plants derived from seeds with entire cotyledon. This result shows that the cotyledons are essential for cashew plants growth after the germination phase. This might be due to adequate nutrient reserves stored in the cotyledon of the seeds with entire cotyledon and seeds type with half cotyledon with the embryonic axis explants having little stored nutrient reserves.

CONCLUSION

The overall objective of this investigation was to define optimal conditions for *in vitro* seeds germination and plant growth of cashew nut. The main results showed that the explant type did not influence the germination parameters but did influence plant viability and growth parameters. The highest normal plants rate was observed with embryonic axis. On the other hand, the best seedling growth is observed with the seeds with half cotyledon followed by seeds with entire cotyledon and the embryonic axis.

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