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Full Length Research Paper

In vitro regeneration of hybrid plantlets of cashew (*Anacardium occidentale* l) through embryo culture

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Embryos from immature nuts of cashew (*Anacardium occidentale* L.) were cultured *in vitro* to regenerate improved hybrid plantlets. Explants (embryo) were excised from developing F1 hybrid immature nuts derived from diallel cross and harvested at 2-, 4-, 6- and 8-weeks after pollination (WAPo) for *in vitro* culture. The explants were surface sterilized, aseptically dissected and cultured into pure basal Murashige and Skoog (MS) agar medium and MS medium supplemented with 1 µM each of naphthaleneacetic acid (NAA), benzyladenine (BA) and gibberellic acid (GA_3) and subsequently observed for germination and survival rates until successful ones were transferred to the field. Age of explants was found to significantly influence both the germination and survival rates. Explants of 6 weeks old and above were found to give better germination rate and highest survival percentage in this study. Only MS medium supplemented with 1 µM of gibberellic acid (MS+GA_3) supported germination and growth at 2-WAPo, suggesting the essentiality of GA_3 as a growth regulator to a very young cashew embryo. Analysis also showed that factors such as medium composition, age of embryo and genotype (accession) significantly influence the germination rate of cashew embryo. It was observed that cashew embryos were found to be autonomy of growth regulator as the age increases and medium composition is only critical at very young age of the embryo. Successful germinated explants simultaneously produced shoot and root and were ready for transfer to field and acclimatization, between 90 and 112 days after inoculation.

Key words: *Anacardium occidentale*, *in vitro* culture, explant, embryo.

INTRODUCTION

Cashew (*Anacardium occidentale* L.) is an important cash crop of the tropics that was introduced into Nigeria in the 15 and 16th centuries and became important plantation crop in 1950s' (Woodroof, 1967; Sanwo, 1972; Venkataramah, 1976; Togun, 1977). Recent survey carried out by Cocoa Research Institute of Nigeria (CRIN) and BioHybrids Agri-Sysytem (U.K.) revealed that the crop thrived in all agro-ecological regions of the country and that much more hectarages of cashew plantations had been established between 1995 and 2000 (Topper et al., 2001). Ayodele et al. (2001) reported that about 100,000 hectares of land has been put to cultivation of cashew in Nigeria as at the year 2000 while annual production stands at 70,000 metric tons. An increasing awareness of the abundant economic potentials of cashew and increase demand of cashew kernels in the global market, has further led to the influx of farmers, government agencies, non-government organizations (NGO) into the agricultural business of cashew production, with the total hectarage of land planted to cashew reaching about 320,000 hectares and about 256,000 metric tons as annual nut production in the 2003 (Aliyu, 2004a). Cashew production in Nigeria continues to be limited by low and variable nut yield, nut quality and susceptibility to pests and diseases (Aliyu, 2004b).

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Table 1. List of 10 selected cashew accessions and their morpho-genetic attributes.

Accession no.	Original source	Present location	Weight of whole fruit (g)	Nut weight (g)	Number of nuts/tree	Apple colour
CSI58	WNDC Iwo	CRIN, Ibadan	64.92	6.43	4920.40	Yellow
CSI 18	"	"	38.89	3.42	2882.30	Yellow
CSI 66	"	"	98.86	6.09	3685.00	Red
CC 05	Ochaja	"	120.90	6.10	3814.00	Red
CC 06	"	"	126.60	7.05	4636.30	Red
CC 11	"	"	24.92	2.88	3685.30	Yellow
CSO 03	Brazil	Oro, Kwara State	39.27	5.53	3539.40	Red
CSO 16	"	"	38.81	3.57	2961.70	Deep Red
CSO 23	"	"	158.78	18.25	347.70	Red
CSO 25	"	"	210.29	16.38	243.60	Yellow

WNDC: Western Nigeria Development Corporation.

However, progress in the improvement of this crop species through conventional breeding methods especially in the area of production of improved hybrid materials has been hampered because of problems of incompatibility, pests and diseases, fire hazards, and other biotic and abiotic factors. Therefore there is need to attempt circumventing these adverse factors through *in vitro* culture of embryos from immature nuts of cashew.

The application of *in vitro* technologies has been employed for a large variety of trees, mostly temperate species (Kannan and Jasrai, 1996). Persely (1992) stressed the need for the applications of biotechniques to many tropical crops with a view to resolving constraints to their productivity. However, successful application of tissue culture system depends solely on a judicious choice of variables including the explant type (Holme and Peterson, 1996); developmental stage and size (Eapen and George; 1999), growth medium, culture condition, and growth regulator among others (Brown, 1990). The optimization of such factors had led to successful cultures in many species (Xiao et al., 1997; Obembe et al., 1999).

Review of the literatures had shown that there has been limited effort in the application of tissue culture techniques to the genetic improvement of cashew and the report to date on the recovery of cashew hybrid seedlings from immature embryos is very scanty. Das et al. (1996) however, reported that even though crops closely related to cashew have been propagated *in vitro*, satisfactory results have not been achieved for cashew. Mantel et al. (1997) also remarked that cashew is strongly recalcitrant to *in vitro* culture techniques and only limited success has been achieved. However, this technique has potential to contribute significantly to the genetic improvement of cashew especially to overcome post-fertilization barriers (incompatibility and pests and diseases) which often resulted into eventual death of the embryos and loss of the hybrid nuts. Occurrence of sterility due to seed non-viability and seedling lethality has also been reported in some species closely related to cashew. This work is therefore initiated to regenerate hybrid plantlets of cashew *in vitro* through embryo culture in order to facilitate and guarantee regular production of improved and hybrid cashew seedlings to farmers.

MATERIALS AND METHODS

Ten accessions of cashew were selected comprising of local and exotic Brazilian genotypes for hybridization. Table 1 showed the list of the accessions and their morphogenetic attributes. Diallel cross was carried out among the 10 selected accessions between November 2001 and March 2002 and the resultant F1 hybrid immature nuts were harvested at 2-, 4-, 6-, and 8-weeks after pollination (WAPo) for *in vitro* culture. Twenty immature hybrid embryos were selected for each developing age and also replicated thrice. The explants were classified on the basis of embryo age from the date of pollination (WAPo), rather than the size in metric, because of fragility of embryos and more importantly to reduce level of contamination (Aliyu and Hammed, 2000).The immature nuts were dipped in 70% ethanol for 1 min, flamed and surface sterilized with 0.1% mercuric chloride for 1 h. Nuts were then washed with sterile distilled water, flamed after dipping in 70% alcohol, cut open aseptically, and the entire seed dissected to remove the embryo. The whitish embryos were cultured *in vitro* in pure basal Murashige and Skoog (1962) (MS) medium and MS medium supplemented with naphthaleneacetic acid (NAA, 1 μM) (MS+NA), benzyladenine (BA, 1 μM) (MS+BA) and gibberellic acid (GA_3, 1 μM) (MS+GA_3) (Mukherjee et al., 1991). The pH was adjusted to 5.8 prior to the addition of 1% activated charcoal and 0.8% agar. Cultured explants were then incubated at 26±2°C with a 16-h photoperiod (Das et al., 1996) and 70-75% relative humidity (Mukherjee et al., 1991). Irradiance at the level of the cultures was given by cool white fluorescent tubes. Successful cultures were transferred to fresh media 30 days after initial inoculation. Percentage success was scored 4 weeks after culture. Data collected were statistically analyzed and results presented in the tables.

Table 2. Effect of age of explants on the response of cashew hybrid embryos to different composition of culture media.

Age of explants (WAPo)	MS	MS+GA_3	MS+NAA	MS+BA
2	0.00 (0.00%)	5.91 (9.85%)	0.00 (0.00%)	0.00 (0.00%)
4	12.99 (21.65%)	19.71 (32.85%)	14.79 (24.65%)	15.69 (26.15%)
6	37.20 (62.00%)	41.31 (68.85%)	36.09 (60.15%)	36.81 (61.35%)
8	45.69 (76.15%)	48.69 (81.15%)	43.20 (72.00%)	42.30 (70.50%)
DMRT $\overline{(x)}$	7.99^{b}	9.63^{a}	7.88^{b}	7.85^{b}

Percentage in parenthesis LSD 0.05 = 0.332.

Table 3. Analysis of variance (ANOVA) of growth and response of cashew hybrid embryos to culture media.

Source of variation	d.f.	SS	MS	F	P
Main – plot analysis					
Replication	2	6.12	3.06		
Embryo age	3	16006.32	5335.44	2400.95	.0000***
Main plot error	6	13.33	2.22		
Subplots analysis					
Accession	9	271.88	30.21	12.89	.0000***
Accession x Embryo age	27	176.03	6.52	2.78	.0003***
Sub-plot error	72	168.72	2.34		
Sub-sub plot analysis					
Medium	3	269.12	89.71	59.50	.0000***
Medium x Embryo Age	9	36.37	4.04	2.68	.0055**
Medium x Accession	27	86.56	3.21	2.13	.0015**
Medium x Accession x Embryo Age	81	129.36	1.60	1.06	3639^{ns}
Error	240	361.83	1.51		
Total	479	17525.65			

Table 4. Mean performance of the 10 selected cashew accessions *in vitro* culture.

Accession No.	2-WAPo	4-WApo	6-WAPo	8-WAPo
CSI 58	0.33	4.33	13.08	14..83
CSI 18	0.08	4.33	12.25	15.75
CSI 66	0.25	4.17	12.08	15.42
CC 05	0.42	6.58	14.17	16.08
CC 06	0.67	8.58	14.75	16.83
CC 11	0.58	4.33	11.50	14.17
CSO 03	0.92	5.17	12.25	14.58
CSO 16	0.75	5.42	11.92	13.50
CSO 23	0.58	5.33	12.17	13.83
CSO 25	0.25	4.42	12.00	14.42
Min.	0.08	4.17	11.50	13.50
Max.	0.92	8.58	14.75	16.83
Mean	0.48	5.23	12.62	14.94
C.V. (%)	54.17	26.58	8.40	7.10

WAPO weeks after pollination.

RESULTS AND DISCUSSION

Table 2 showed the germination and survival rates of cashew hybrid embryo explants of four age categories to different culture media. It was observed that age of explants significantly influence the germination and survival rates. Initially, it was observed that explants of all the four age categories showed callusing with translucent white colour of the embryo gradually changed to green and evidence of growth was noticed within 14-21 days of

inoculation. However, all explants in age 2-WAPo except few (9.85%) growing on the MS+GA_3 medium remain in this condition for four weeks without further growth after which they turned brown and died back. The germination and survival rate of 9.85% recorded in the explants of 2-WAPo growing in MS+GA_3 culture medium indicates its suitability, essentiality and comparative advantage over other three media composition in this study. The trend of MS+GA_3 superior performance was observed with explants of other age categories (Table 2). However, the poor germination recorded for the explants of 2-WAPo is suggesting that the resultant fertilized zygote of cashew may still be undergoing embryogenic development at this stage of nut development in cashew. This is likely responsible for the death of majority of the embryos of the 2-week old that died at 4 weeks of inoculation except those on MS+GA_3 medium. However, Aliyu and Hammed (2000) reported third and fourth weeks after pollination (3-WApo and 4-WAPo) as a period for complete development of cashew embryo. Therefore the improve response of 4-WAPo explants with germination rate ranging from 21.65 to 32.855%, however, attest to the findings of these authors and indicates that embryo of 4-week old is more physiogenetically mature than the 2-week old ones and therefore more suitable for *in vitro* culture in cashew.

Explants of 6-WAPo and above, respond positively to all the four media composition and gave better germination (Table 2). Germination rates ranging from 62.00 to 81.15% were recorded among the 6-WAPo and 8-WAPo explants. However, Ohler (1979) and Aliyu and Hammed (2000) reported that cashew attains complete physiological maturity at the 6-WAPo. It is perhaps implies that the significant difference observed between the performance of explants at 4-WAPo and 6-WAPo may be attributed to the physiological maturity of embryo at that stage of cashew nut development and therefore making it amenable to culture readily. Comparatively, MS+GA_3 medium consistently ranked first among the media composition tested in this study, while the performance of explants on pure MS, MS+NA and MS+BA was not significantly different from each other. Table 3 showed the combine analysis of variance of all the factors (embryo age, medium and accession). The main analysis showed that there was no significant difference due to replication, but age of embryo was highly significantly different at $P<0.0001$ probability level. This is suggesting that variability in the age of explants would significantly affect the rate of performance in the culture. In the sub-plot analysis, highly significant differences were recorded for accession (genotype) and its interaction with age of embryo. Variation in the performance of the explants was also found to be highly significant ($P<0.0001$) for medium, significant at $P<0.0055$ and $P<0.015$ for the interaction between medium x age of embryo and medium x accession respectively in the sub-sub-plot analysis. However, interaction among these three factors (age of embryo, accession and medium) was not significant. It is implying that these factors either singly or in combination influence the success rate of *in vitro* plantlets regeneration in cashew.

The study revealed that pure MS, MS+NAA and MS+BA media are capable of supporting the growth of cashew hybrid embryos of 4-WAPo and above effectively. The successful germination of embryos of 4-WAPo and above in growth regulator free (pure MS) medium (Table 2), indicates that exogenous growth regulators are not too essential for the germination, growth and development of cashew embryo of that age onward. However, among all the growth regulators studied, GA_3 was found most essential for initial morphogenesis of cashew embryo. Mukherjee et al. (1991) also reported essentiality of GA_3 for initial morphogenesis of sweet potato embryo. Raghavan (1966) well defined the nutrition, growth and morphogenesis of globular and post-globular embryos in culture. This study shows similar trend of increasing autonomy of embryo with age.

The accessions responded differently to the culture and this resulted to significant result obtained in the analysis of variance (ANOVA) (Table 3). CC05 and CC06 accessions were found to be the genotypes with the best morphogenic potential among those evaluated as they consistently gave higher germination rate than the others (Table 4). However, variation (c.v.) in the germination performance among the accessions (genotypes) was very high for the 2-WAPo explants (54.17%), but consistently decrease as the age of embryo increases (Table 4). This however indicates that effect of genotypes was more pronounced on the younger embryos than the older ones. This perhaps may be attributed to physio-genetic characteristics and degree of compatibility of the maternal parents. Aliyu (2004b) recorded highest compatibility and fruit-set in these two accessions (CC05 and CC06) in previous work on compatibility study in cashew. It was also observed that the response of 2-WAPo embryos highly varied with c.v of 196.00% and this was as a result of non-germination of many explants of this age in most of the media except MS+GA_3. Trend of embryo response and germination to different media composition was similar to that of genotype (accession), because as the age of embryo increase the extent of variability due to media composition reduces significantly (Table 5). The variations due to addition of growth hormones among the 6- and 8-WAPo explants were just about 6.18 and 6.37%, respectively. This also implies that medium composition is more critical to younger embryo in cashew than the mature ones. Mofidabadi and Modir-Rahmati (2000) observed that embryo age affected the ability of the embryos to respond to culture media in *Populus euphratic* Oliv. and *Populus alba* L.. This observation also emphasize the autonomy of cashew embryo with age and was similar to the report of Raquin

Table 5. Mean germination of explants of different developing ages.

Medium	2-WAPo	4-WAPo	6-WAPo	8-WAPo
MS	0.00	4.33	12.40	15.23
MS+GA_3	1.97	6.57	13.77	16.23
MS+NAA	0.03	4.93	12.03	14.40
MS+BA	0.00	5.23	12.27	14.10
Min.	0.00	4.33	12.03	14.10
Max.	1.97	6.57	13.77	16.23
Mean	0.50	5.26	12.62	14.99
C.V. (%)	196.00	18.06	6.18	6.37

WAPo, Weeks after pollination; C.V., Coefficient of variation.

Table 6. Survival rates of plantlets during subsequent sub-culturing using MS+GA3 medium.

Explant age	28DAI	56DAI	84DAI	112DAI
2	5.91(9.85)	3.80 (6.33)	3.20 (5.33)	3.20 (5.33)
4	19.71 (32.85)	18.00 (30.00)	17.90 (29.83)	17.90 (29.83)
6	41.31 (68.85)	40.25 (67.08)	40.25 (67.08)	40.00 (66.67)
8	48.69 (81.19)	48.60 (81.00)	48.50 (80.83)	48.00 (80.00)

Percentage in parenthesis DAI, Days after inoculation.MS+GA_3: MS medium supplemented with GA_3.

Figure 1. 90 days old hybrid cashew plantlets *in vitro* regenerated through embryo culture.

and Troussard (1993) that successfully generated plantlets from *Populus* spp. embryos cultured in only MS medium containing only mineral salts, water, sucrose and no growth regulator. Very low germination recorded among the embryos of 2-WAPo was also similar to the report of these authors on *Populus* spp. The authors observed that embryo younger than 30 days (10 and 20 days old) were reported not to respond to the culture media.

Table 6 showed the survival rate of successfully germinated plantlets over three subcultures before acclimatization and final transfer to field as seedlings. Figure 1 showed 90-day old hybrid cashew plantlets growing in the cultured bottle. It was clearly evident that the survival percentage after successful germination was consistently stable among older embryos (4- weeks old and above) than the younger ones. About 54.11% of successful germinated plantlets of 2-WAPo were finally transferred to field at 112 days after inoculation, while 86.25, 96.83 and 98.58% survival/transfer rates were achieved for plantlets derived from 4-, 6- and 8- weeks old embryos, respectively. Generally, it was observed that in successful germinated explants, irrespective of age, both root and shoot development simultaneously took place from the radicle and plumule, respectively, at the same time and at a faster rate when compared to slow growth that was reported for *Cola* species (Adebola, 2003). Direct regeneration of rooted plantlets from the embryos cultured on media with growth regulators in this work is at variance to the observations reported by Kouider et al. (1984) and Li and Li (1985). The authors observed that when embryos were culture on media based on MS components supplemented with growth hormones, 3-indole-acetic acid (IAA) and 6-benzylaminopurine (BAP), it resulted in the production of calluses and multiple shoots and it was necessary to transfer to rooting medium before plantlets were obtained. In this study, however, cashew embryos of right

age (4- and 8-WAPo) successful develop to plantlet both on MS without and MS with growth hormones. Mukherjee et al. (1991) also recorded callusing and multiple shoot development sweet potato (*Ipomea batata* L.) embryos when three growth regulators (1 µM NAA + 2 µM BA + 0.5 µM GA_3) were added to MS.

In summary, the study has thrown light on spectrum of the potential abound in the *in vitro* regeneration of hybrid plantlets of cashew using embryo from immature nuts as explants. It was clearly demonstrated in this work that embryo of 4-weeks old and above can be successfully cultured on MS basal medium with or without growth regulator(s). However, addition of growth regulator such as gibberellic acid (GA_3) becomes highly imperative when using a much younger embryo. The study equally showed that production of improved hybrid cashew via *in vitro* embryo culture is a task that can be easily accomplished and there must be widely adopted especially in countries with severe attack of pests and diseases. Although direct regeneration of plantlets from embryos recorded in this study is of great importance and success in the recovery or regeneration of hybrid seedlings both in inter- and intra- specific crosses of cashew, however, the formation of multiple shoots from a single embryo could be achieved through further studies on combination of different growth regulator with MS medium using very young embryo especially 2-WAPo old. Effort in this direction would be of great importance in the multiplication of hybrid embryos – plantlets of cashew in future.

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