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Induction of Embryogenic Callus in Cashew (*Anacardium occidentale* L.) by Cotyledon, Nucellus and Testa with Antioxidants Effect

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Authors' contributions

This work was carried out in collaboration among all authors. Authors BSAA, BTB, SSH conceptualized the topic and investigated the work while authors BSAA, GHTC, BTB written first original draft. Authors CA and JD complementally supervised the work and JAH have reviewed and edited the manuscript for publication. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This work aims to evaluate the influence of different growth regulators combined with antioxidants on the induction of callus with different explants in cashew.
Materials and Methods: Nucellar tissues, cotyledons and testa from elite tree nuts are cultured on media that differ in their concentration of 6-Benzylaminopurine (BAP: 0 mg/l; 0.25 mg/l; 0.5 mg/l) and Acid 2,4 dichlorophenoxyacetic (2,4-D: 0 mg/l; 0.8 mg/l). Therefore, for the control of the browning of the explants, the effect of activated charcoal and polyvinylpyrrolidone was tested. The amount of callus formed is evaluated after 28 days and after 90 days by simple observation according to a given scale.
Results: Analysis of variance of callus formation 28 days after the culture of explants shows that the interaction between growth regulators and antioxidants significantly influences ($p < 0.05$) the induction of callus. The combination BAP 0.25mg/l and 2,4-D 0.8mg/l produces on average more

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callus (0.47 ± 0.00). There is a very significant difference (P < 0.05 to p<0.001) in the effect of growth regulators and antioxidants on obtaining callus after 90 days of cultivation. The 0.25 mg/l combination of BAP and 0.8 mg/l of 2,4-D still appears to be the best combination of growth regulators for callus with 58% of callus formed. Cotyledons in the presence of PVP respond better than activated charcoal. The nucellus are the explants that respond better in the presence of activated charcoal. The test for Least Significant Difference reveals that PVP significantly promotes (p <0.05) the production of a large number of calli (77%) unlike activated charcoal (9%) after 90 days of culture.

Conclusion: Summary, for obtaining viable and embryogenic callus in cashew, the combination of BAP at 0.25 mg/L and 0.8 mg/L of 2,4-D is the best and this in the presence of PVP with cotyledons.

Keywords: Anacardium occidentale; callus; 6-Benzylaminopurine; antioxidant; embryogenesis.

ABBREVIATIONS

AC : Activated Charcoal
ANOVA : Analysis of Variance
BAP : 6-benzylaminopurine
B5 : Gamborg medium
°C : Degree Celsius
2,4-D : 2,4-Dichlorophenoxiacetic acid
g/l : gramme per litre
IAA : Indole-3-Acetic Acid
LSD : Least Significant Difference
mg/l : milligram per litre
mm : millimetre
MS : Murashige and Skoog, 1962
% : Percentage
PEC : Pro-Embryonic Calli
PGR : Plant Growth Regulators
pH : Potential of Hydrogen
PVP : Polyvinylpyrrolidone
SAS : Statistical Analysis System

1. INTRODUCTION

Anacardium occidentale L. is an agriculturally important crop in several tropical regions such as Asia, Africa, India and Latin America. The cashew is highly valued for its edible nuts and shell. Conventional plant breeders and biotechnology programmes to improve productivity are giving it great interest. The growing demand for cashew kernels has led to an increase in the cultivation of this plant. However, current propagation methods have become a limiting factor in the supply of adequate planting material [1]. Conventional methods of vegetative propagation, namely layering, grafting or cutting, are not fast to meet the needs of elite varieties in a timely manner. The technology for commercial-scale propagation of plants is mainly based on micropropagation. Micropropagation, especially somatic embryogenesis protocols, aims at rapid multiplication of seedlings true to the parent material [2]. Although the successes of somatic embryogenesis have been documented for some highly productive forest and fruit species, there are still barriers to the implementation of somatic embryogenesis on an operational scale in forestry and agroforestry [3]. Among other constraints to cashew micropropagation, explant browning and the type of growth regulator are the main factors to be controlled in the induction of somatic embryogenesis [4]. In *in vitro* culture, cashew, like other Anacardiaceae, is very recalcitrant and only limited success has been achieved [4,5]. Several studies have shown in cashew and mango that 2,4-D is the essential component of the induction phase of somatic embryogenesis in several species. However, the prolonged presence of 2,4-D in the induction medium inhibits the growth of somatic embryos beyond the globular stage [6,7]. Recent studies in mango [8], a species of the same family as cashew, have shown that improved embryo production is observed when the concentration of nitrogen in the medium, especially ammonium salts, is low and 2,4-D is replaced by BAP at low concentration (0.25 mg/l). Furthermore, cashew is rich in phenolic compounds. Mantell et al [5] report that secondary metabolites are released in the phloem vessels of all cashew organs. Indeed, the wounds caused during the cutting of explants favour the oxidation of these phenolic compounds, whose exudation leads to the browning and then the necrosis of the calli. Thus, a low rate of calli is obtained at the end of the induction. Establishing an effective protocol to control browning is therefore a challenge. Cultivation of explants with antioxidants such as 0.3% Polyvinylpyrrolidone (PVP) [9] combined with frequent subculturing and dark incubation [10], addition of activated charcoal to the basal medium [11], minimize the effect of phenolic exudations and consequently, explant necrosis. Thus, the aim of this work is to evaluate the effect of BAP and 2,4-D in combination with PVP or activated charcoal in the formation of pro-embryogenic calli in order to obtain somatic

embryos capable of germinating and regenerating the whole plant of *Anacardium occidentale in vitro*.

2. MATERIALS AND METHODS

2.1 Materials

Immature seeds (2-3 weeks after fertilization) collected on selected cashew trees from the municipality of Bassila retained by previous studies were surface sterilized for 5 min in 70% (v/v) ethanol, followed by 50% sodium hypochlorite (4% active chlorine) solution with a few drops of Tween-20 for 30 minutes; and then rinsed three times in sterile distilled water. Ovules were removed from cashew nut under sterile conditions and bisected longitudinally [12]. The nuts were cut open under sterile conditions in a laminar hood, the ovules were dissected out, cut in half. The immature seed was taken out using sterile forceps and to allow removal of the embryo and nucellus. Thick and fleshy seed coat explants, nucelli and cotyledon were cultured on Modified Gamborg's B5 major salts, MS minor salts, iron source and organics with varying hormone concentrations [8]. The explants were placed on the various media with either their convex (dorsal) or concave (ventral, cut end) side in contact.

2.2 Methods

The medium supplemented with 6% sucrose, 400 mg/L L-glutamine, 500 mg/L casein hydrolysate, 80 mg/l adenine sulphate and, with 0.8 mg/L 2,4-dichlorophenoxy-acetic acid (2,4-D) alone, or in combination with BAP at 0.25 mg/l or 0.5 mg/l (Table 1) giving five treatments in all 2.5 g/l of activated charcoal or PVP. For callus induction, 10–15 explants were used per treatment (explant x media), with three replicates in a randomized block design. All the experiments were repeated twice.

Cultures were incubated in the dark at 25° ± 1°C for 12 weeks, and callus formation was observed each week. After 28 and 90 days, the number of explants showing callus and pro-embryonic calli (PEC) commencement, as well as the number of days required for callus and PEC development, were recorded.

2.3 Statistical Analysis

The data collected after 28 and 90 days were entered and processed with the Excel spreadsheet. These data were then subjected to a two and three-way analysis of variance (ANOVA) using the PROC GLM procedure of the Statistical Analysis System (SAS) software version 9.2. Data for the amounts of calli after four weeks were subjected to a three-way ANOVA (growth regulators, antioxidants and explants) while the data taken after 90 days were subjected to a two-way ANOVA (growth regulators and antioxidant). Multiple means comparisons were made with the test for the least significant difference (LSD) at the 5% level [13].

Table 1. Media compositions for the induction of calli from the various types of explants

Media	BAP (mg/l)	2,4-D (mg/l)	PVP (g/l)	Activated charcoal (g/l)
C0P	0	0.8	2.5	0
C1P	0.25	0	2.5	0
C2P	0.5	0	2.5	0
C3P	0.25	0.8	2.5	0
C4P	0.5	0.8	2.5	0
C0ch	0	0.8	0	2.5
C1ch	0.25	0	0	2.5
C2ch	0.5	0	0	2.5
C3ch	0.25	0.8	0	2.5
C4ch	0.5	0.8	0	2.5

The amount of callus is evaluated using the scale: no callus = 0 cal; slight callus = 1mm cal; moderate callus = 2 to 5mm; profuse callus = 5 to 8mm; highly profuse callus = 8 to 10mm) is noted after 4 weeks and after 90 days by simple observation

3. RESULTS

3.1 Morphological Response

Morphological changes such as swelling, color change were noticed from the first week of culture. Regardless of the treatment, 98% of the explants showed morphological responses. After the first week of culture, the cotyledons turned green and tripled or even quadrupled in size after three weeks of culture. The explants reacted differently and depending on the composition of the medium. In the cotyledons, calli were observed on the edge and on the outer side. In the nucellus, calli were observed on the surface above the medium. The testa gave some calli and this on the outer layer. The testa (Fig. E) rapidly turned brown and necrotic (Fig. F). The cotyledons and the nucellus were the best explants to obtain the callus. The nucellar callus was brown (Fig. D), while the cotyledonary callus was snow white, translucent, white crystals or green (Fig. B).

3.2 Callus Formation

The three-factor analysis of variance considering media, antioxidants and explants on callus formation after four weeks of cultivation revealed (Table 2) that there was no significant difference ($p>0.05$) between plant growth regulators and antioxidants with respect to callus formation. However, the interaction between hormone composition and antioxidants significantly ($p<0.05$) influenced callus induction. Table 3 shows the results of the smallest significant difference test. From this table it can be seen that the testa did not significantly ($p>0.05$) induce callus formation compared to the nucellus and cotyledon, which did induce callus and even of medium size. Activated charcoal appeared to be the antioxidant that significantly ($p<0.05$) promoted callus formation in the medium with only 2,4-D at 0.8 mg/l (C0) and the medium with BAP at 0.25 mg/l and no 2,4-D (C1). The medium (C1) was less favourable for callus formation. However, media C0 where BAP was zero (0mg/l) and 2,4-D 0.8mg/l; then C3 where BAP was low (0.25mg/l) and 2,4-D 0.8mg/l produced significantly ($p < 0.05$) more callus (0.47 ±0.00 on average) according to the Least Significant Difference test. In general, C0, C4 and C3 media were the best for obtaining average callus with 46%, 37% and 23% average callus respectively. It was also noted that activated carbon gave more callus on media C0 and C1. In general, it was found that media C0 where BAP was zero (0mg/l) and 2,4-D 0.8mg/l; then C3 where BAP was low (0.25mg/l) and 2,4-D 0.8mg/l produced more callus (0.47 ± 0.00 on average).

Table 2. F-values and level of significance from three-way Analysis of Variance (ANOVA) of callus formation regarding plant growth regulators (PGR), antioxidant and explant after 28 days

Source of variation	Degree of freedom	F-values				
		Callus formation after 28 days				
		No callus	slight callus	moderate callus	Profuse callus	Highly profuse callus
PGR	4	0.85^{ns}	0.69^{ns}	1.03^{ns}	2.58^{ns}	0.12^{ns}
Antioxidants	1	0.09^{ns}	0.55^{ns}	0.05^{ns}	0.90^{ns}	0.20^{ns}
Explants	2	3.06^{ns}	0.64^{ns}	1.83^{ns}	1.7^{ns}	0.28^{ns}
PGR * Antioxidants	4	3.63*	0.73^{ns}	3.11^{*}	0.72^{ns}	0.12^{ns}
PGR * Explants	8	0.64^{ns}	0.70^{ns}	0.63^{ns}	1.8^{ns}	0.11^{ns}
Antioxidants * Explants	2	0.50^{ns}	0.72^{ns}	0.34^{ns}	0.62^{ns}	0.28^{ns}
PGR* Antioxidants*Explants	8	2.04^{ns}	0.77^{ns}	1.06^{ns}	1.04^{ns}	0.13^{ns}

*ns: P >0.05 *: P < 0.05*

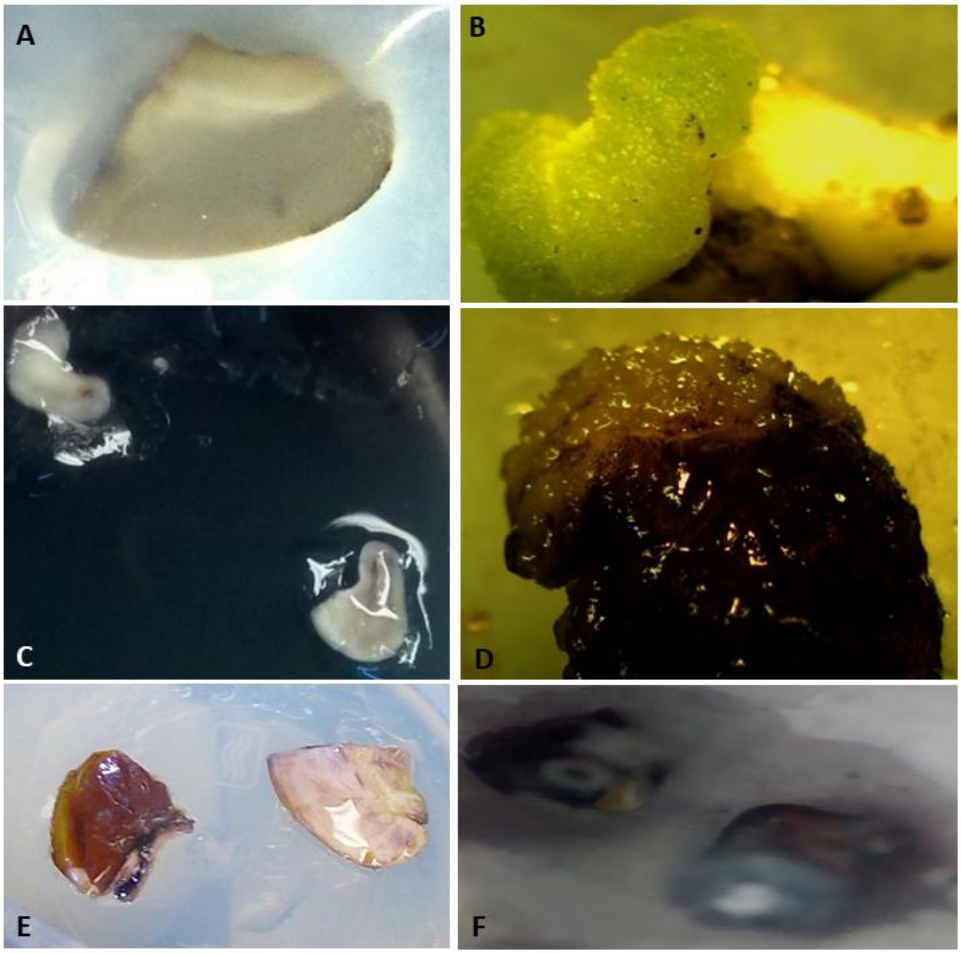

Fig. 1. Morphological response of different explants on the medium
A: Cotyledon explant; B: Calli from Cotyledon; C: Nucellar explant; D: Calli from Nucellar; E: Testa explant; F: Necrosis of testa explant

Table 4 presents the results of the analysis of variance after 90 days of initial culture of the explants. The analysis of the table showed that there was a highly significant difference ($P < 0.05$ to $p < 0.001$) in the effect of growth regulators and antioxidants on calli production. There was also a significant effect of growth regulators on the achievement of low calli. The interaction between growth regulators and antioxidants was significant in relation to calli production. Furthermore, it was observed that the formation of moderate profuse, and highly profuse callus was not significantly different ($p > 0.05$) between the media and antioxidants. Table 5 shows the effect of growth regulators and antioxidants on callus formation. The Least significant difference test revealed that PVP significantly ($p < 0.05$) favored a large number of calli (77%) in contrast to activated carbon (9%) on the medium with BAP 0.25 mg/l and 2,4-D 0.8 mg/l (C3). Similarly, C3 medium with the combination of BAP 0.25 mg/l and 0.8 mg/l 2,4-D on the one hand and C4 medium with BAP 0.5 mg/l and 2,4-D 0.8 mg/l on the other hand appeared to be the best media for obtaining callus with 58% and 45% of callus formed respectively. In most cases, the combination of 0.25 mg/l BAP and 0.8 mg/l 2,4D (C3) in the presence of PVP was significantly ($p < 0.05$) better for callus formation.

3.2.1 Effect of the concentration of growth regulators on callus formation

Table 6 shows the effect of concentration of growth regulators and type of antioxidant on callus formation. It was noted that the existence of both growth regulators in the medium is important for callogenesis in cashew. The C3 medium: 0.25 mg/l BAP and 0.8 mg/l 2,4-D seemed to be the most important regulators of callogenesis.

Table 3. Effect of growth regulators, antioxidant and explant on callus formation after 28 days (mean ± standard error)

Media	Antioxidants	Explants	Callus formation				
			No callus	slight callus	moderate callus	Profuse callus	highly profuse callus
BAP 0mg/l and 2,4-D 0,8mg/l (C0)	Activated charcoal	Nucellus	0,00±0,00b	0,00±0,00a	1,00±0,00a	0,00±0,00a	0,00±0,00a
		Testa	1,00±0,00a	0,00±0,00a	0,00±0,00b	0,00±0,00a	0,00±0,00a
		Cotyledon	0,00±0,00b	0,00±0,00a	1,00±0,00a	0,00±0,00a	0,00±0,00a
		Mean	0,20±0,20B	0,00±0,00A	0,80±0,20A	0,00±0,00A	0,00±0,00A
	PVP	Nucellus	1,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a
		Testa	1,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a
		Cotyledon	0,50±0,28a	0,00±0,00a	0,50±0,28a	0,00±0,00a	0,00±0,00a
		Mean	0,750±0,16A	0,00±0,00A	0,25±0,16B	0,00±0,00A	0,00±0,00A
	Mean		0,53±0,00Y	0,00±0,00X	0,46±0,00X	0,00±0,00X	0,00±0,00X
BAP 0,25 mg/l and 2,4-D 0mg/l (C1)	Activated charcoal	Nucellus	1,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a
		Testa	1,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a
		Cotyledon	0,00±0,00a	0,00±0,00a	1,00±0,00a	0,00±0,00a	0,00±0,00a
		Mean	0,66±0,33B	0,00±0,00A	0,33±0,33A	0,00±0,00A	0,00±0,00A
	PVP	Nucellus	1,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a
		Testa	1,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a
		Cotyledon	1,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a
		Mean	1,00±0,00A	0,00±0,00A	0,00±0,00B	0,00±0,00A	0,00±0,00A
	Mean		0,90±0,00X	0,00±0,00X	0,10±0,00Y	0,00±0,00X	0,00±0,00X
BAP 0,5mg/l and 2,4-D 0mg/l (C2)	Activated charcoal	Nucellus	1,00±0,00a	0,00±0,00a	0,00±0,00 a	0,00±0,00 a	0,00±0,00 a
		Testa	1,00±0,00a	0,00±0,00a	0,00±0,00 a	0,00±0,00 a	0,00±0,00 a
		Cotyledon	1,00±0,00a	0,00±0,00a	0,00±0,00 a	0,00±0,00 a	0,00±0,00 a
		Mean	1,00±0,00A	0,00±0,00A	0,00±0,00A	0,00±0,00A	0,00±0,00A
	PVP	Nucellus	0,50±0,50 a	0,00±0,00 a	0,50±0,50 a	0,00±0,00 a	0,00±0,00 a
		Testa	1,00±0,00 a	0,00±0,00 a	0,00±0,00 a	0,00±0,00 a	0,00±0,00 a
		Cotyledon	0,60±0,24 a	0,00±0,00 a	0,20±0,20 a	0,00±0,00 a	0,20±0,20 a
		Mean	0,70±0,15A	0,00±0,00A	0,20±0,13A	0,00±0,00A	0,10±0,10A
	Mean		0,78±0,00X,Y	0,00±0,00X	0,14±0,00X,Y	0,00±0,00X	0,07±0,00X

BAP 0,25mg/l and 2,4-D 0,8mg/l (C3)	Activated charcoal	Nucellus	0,00±0,00b	0,50±0,50 a	0,50±0,50 a	0,00±0,00 a	0,00±0,00 a
		Testa	1,00±0,00 a	0,00±0,00 a	0,00±0,00 a	0,00±0,00 a	0,00±0,00 a
		Cotyledon	1,00±0,00 a	0,00±0,00 a	0,00±0,00 a	0,00±0,00 a	0,00±0,00 a
		Mean	0,50±0,28A	0,25±0,25A	0,25±0,25A	0,00±0,00A	0,00±0,00A
	PVP	Nucellus	1,00±0,00 a	0,00±0,00 a	0,00±0,00a	0,00±0,00a	0,00±0,00a
		Testa	1,00±0,00 a	0,00±0,00 a	0,00±0,00a	0,00±0,00a	0,00±0,00a
		Cotyledon	0,20±0,20 a	0,00±0,00 a	0,40±0,24a	0,00±0,00a	0,40±0,24a
		Mean	0,55±0,17A	0,00±0,00A	0,22±0,14A	0,00±0,00A	0,22±0,14A
	Mean		0,53±0,00Y	0,07±0,00X	0,23±0,00X,Y	0,00±0,00X	0,15±0,00X
BAP 0,5mg/l and 2,4-D 0,8mg/l (C4)	Activated charcoal	Nucellus	1,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a
		Testa	1,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a
		Cotyledon	1,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a
		Mean	1,00±0,00A	0,00±0,00A	0,00±0,00A	0,00±0,00A	0,00±0,00A
	PVP	Nucellus	0,00±0,00a	0,00±0,00a	1,00±0,00a	0,00±0,00a	0,00±0,00a
		Testa	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a
		Cotyledon	0,33±0,33a	0,00±0,00a	0,66±0,33a	0,00±0,00a	0,00±0,00a
		Mean	0,25±0,25A	0,00±0,00A	0,75±0,25A	0,00±0,00A	0,00±0,00A
	Mean		0,62±0,00X,Y	0,00±0,00X	0,37±0,00X,Y	0,00±0,00X	0,00±0,00X

Within column, means followed by letters of same characters are not significantly different (P > 0.05) according to the test for the Least Significant Difference

Table 4. F-values and level of significance from two-way Analysis of Variance (ANOVA) of callus formation regarding plant growth regulators (PGR) and antioxidant after 90 days

Source of variation	Degree of freedom	F-values				
		Callus formation after 90 days				
		No callus	slight callus	moderate callus	Profuse callus	highly profuse callus
PGR	4	5.58^{***}	2.78^{*}	0.80^{ns}	0.75^{ns}	1.85^{ns}
Antioxidants	1	6.43^{*}	0.95^{ns}	0.38^{ns}	0.89^{ns}	1.11^{ns}
PGR* Antioxidants	4	3.43*	1.14^{ns}	0.80^{ns}	0.32^{ns}	1.85^{ns}

*ns: P >0.05 *: P < 0.05; ***: P < 0.001*

Table 5. Effect of growth regulators, antioxidant and explant on callus formation after 90 days (mean ± standard error)

Media	Antioxidants	Callus formation				
		No callus	slight callus	moderate callus	Profuse callus	highly profuse callus
BAP 0mg/l and 2,4-D 0,8mg/l (C0)	Activated charcoal	1,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a
	PVP	1,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a
	Mean	1,00±0,00A	0,00±0,00B	0,00±0,00A	0,00±0,00A	0,00±0,00B
BAP 0,25mg/l and 2,4-D 0mg/l (C1)	Activated charcoal	1,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a
	PVP	1,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a
	Mean	1,00±0,00A	0,00±0,00B	0,00±0,00A	0,00±0,00A	0,00±0,00B
BAP 0,5mg/l and 2,4-D 0mg/l (C2)	Activated charcoal	1,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a
	PVP	0,92±0,07a	0,00±0,00a	0,00±0,00a	0,07±0,07a	0,00±0,00a
	Mean	0,94±0,05A	0,00±0,00B	0,00±0,00A	0,05±0,05A	0,00±0,00B
BAP 0,25mg/l and 2,4-D 0,8mg/l (C3)	Activated charcoal	0,91±0,08a	0,08±0,08b	0,00±0,00a	0,00±0,00a	0,00±0,00a
	PVP	0,23±0,07b	0,40±0,09a	0,16±0,06a	0,20±0,07a	0,00±0,00a
	Mean	0,42±0,07B	0,30±0,07A	0,11±0,05A	0,14±0,05A	0,00±0,00B
BAP 0,5mg/l and 2,4-D 0,8mg/l (C4)	Activated charcoal	0,88±0,11a	0,00±0,00a	0,00±0,00a	0,11±0,11a	0,00±0,00a
	PVP	0,50±0,13a	0,07±0,07a	0,00±0,00a	0,21±0,11a	0,21±0,11a
	Mean	0,65±0,10B	0,04±0,04B	0,00±0,00A	0,17±0,08A	0,13±0,07A

Within column, means followed by letters of same characters are not significantly different (P > 0.05) according to the test for the Least Significant Difference

3.2.2 Combined effect of 2,4-D and antioxidants polyvinylpyrrolidone (pvp) and activated charcoal on the formation and survival of callus

2,4-D was able to induce callus from cotyledons on both activated charcoal and polyvinylpyrrolidone. On the activated charcoal medium, the nucellus also formed calli. However, callus formation was observed 28 days after the initial culture and the calluses did not last over time. Activated charcoal appeared to be the antioxidant that significantly ($p < 0.05$) favoured callus formation on the medium with only 2,4-D at 0.8 mg/l (C0).

3.2.3 Effect of BAP at 0.25 mg/l on callus formation and survival

Only the cotyledons yielded calli with both activated charcoal and polyvinylpyrrolidone with BAP at 0.25 mg/l. These calli appeared after 28 days of initial culture but did not survive.

3.2.4 Combined effect of BAP at 0.5 mg/l and antioxidants (polyvinylpyrrolidone and activated charcoal) on the formation and survival of callus from various explants

BAP at 0.5 mg/l produced callus from nucellus and cotyledons but only with polyvinylpyrrolidone as antioxidant (Table 9). The calli that were produced from the cotyledons lasted up to 90 days, but the rate had halved. On the other hand, with activated charcoal, no callus production was noted for any explant.

3.2.5 Combined effect of BAP at 0.25 mg/l; 2,4-D at 0.8 mg/l and antioxidants polyvinylpyrrolidone and activated charcoal on the formation and survival of callus from various explants

The analysis in Table 10 showed that the combination of BAP at 0.25 mg/l and 2,4-D at 0.8 mg/l produced callus that survived even beyond 90 days. Cotyledons in the presence of polyvinylpyrrolidone produced 2.40±0.29 callus that survived even beyond 90 days after the initial culture. Whereas with activated charcoal, callus formation did not occur after 28 days but we noticed it after 90 days. Nucellus, on the other hand, gave callus in the presence of both antioxidants with a high rate in the presence of activated charcoal (0.60±0.29). Moreover, these calli survived even beyond 90 days.

3.2.6 Combined effect of BAP at 0.5 mg/l, 2,4-D and antioxidant on the formation and survival of callus

Table 11 showed that the presence of 0.5 mg/l BAP, 0.8 mg/l 2,4-D and 2.5 g/l polyvinylpyrrolidone increased the amount of callus with cotyledons. The combined presence of BAP and 2,4-D growth regulators increased the amount of callus formed. On the other hand, with activated charcoal, no callus production was noted for any explant except for nucellus which produced 0.20±0.00 callus after 90 days.

Table 6. Effect of growth regulators on callus formation after 90 days of culture (mean ± standard errors)

Amount of callus formed	BAP 0mg/l et 2,4-D0.8mg/l (C0)	BAP 0.25 mg/l et 2,4-D0mg/l (C1)	BAP 0.5mg/l et 2,4-D0mg/l (C2)	BAP 0.25mg/l et 2,4-D0.8mg/l (C3)	BAP 0.5mg/l et 2,4-D0.8mg/l (C4)
No callus	1.00±0.00a	1.00±0.00a	0.94±0.05a	0.42±0.07b	0.65±0.10b
Slight callus	0.00±0.00b	0.00±0.00b	0.00±0.00a	0.30±0.07a	0.04±0.04b
Moderate callus	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.11±0.05a	0.00±0.00a
Profuse callus	0.00±0.00a	0.00±0.00a	0.05±0.05a	0.14±0.05a	0.17±0.08a
highly profuse callus	0.00±0.00b	0.00±0.00b	0.00±0.00b	0.00±0.00b	0.13±0.07a

The means followed by the same alphabetical letter of the same character and for the same factor are not significantly different (P> 0.05) according to the test for the Least Significant Difference

Table 7. Combined effect of 2,4-D and antioxidants polyvinylpyrrolidone (pvp) and activated charcoal on callus formation and survival (mean ± standard errors)

Explants	Antioxidants	28 days	90 days
Nucellus	Activated charcoal	1.20±0.32a	0.00±0.00a
testa	Activated charcoal	0.00±0.32b	0.00±0.00a
Cotyledon	Activated charcoal	0.50±0.36a	0.00±0.00a
Nucellus	polyvinylpyrrolidone	0.00±0.32b	0.00±0.00a
testa	polyvinylpyrrolidone	0.00±0.32b	0.00±0.00a
Cotyledon	polyvinylpyrrolidone	1.00±0.36a	0.00±0.00a

The means followed by the same alphabetical letter of the same character and for the same factor are not significantly different (P> 0.05) according to the test for the Least Significant Difference

Table 8. Effect of BAP at 0.25 mg/l and antioxidants polyvinylpyrrolidone (pvp) and activated charcoal on callus formation and survival (mean ± standard errors)

Explants	Antioxidants	Callus after 28 days	Callus after 90 days
Cotyledon	polyvinylpyrrolidone	0.800±0.283a	0.00±0.00 a
Nucellus	polyvinylpyrrolidone	0.000±0.00a	0.00±0.00 a
Testa	polyvinylpyrrolidone	0.000±0.00a	0.00±0.00 a
Cotyledon	Activated charcoal	0.800±0.00a	0.00±0.00 a
Nucellus	Activated charcoal	0.000±0.00a	0.00±0.00 a
Testa	Activated charcoal	0.000±0.00a	0.00±0.00 a

The means followed by the same alphabetical letter of the same character and for the same factor are not significantly different (p> 0.05) according to the test for the least significant difference

Table 9. Effect of BAP at 0.5 mg/l and antioxidants polyvinylpyrrolidone (pvp) and activated charcoal on callus formation and survival (mean ± standard errors)

Explants	Antioxidants	Callus (28 days)	Callus (90 days)
Cotyledon	polyvinylpyrrolidone	1.20±0.36a	0.60 ± 0.24a
Nucellus	polyvinylpyrrolidone	0.40±0.36a	0.00± 0.00a
Testa	polyvinylpyrrolidone	0.00±0.00b	0.00± 0.00a
Cotyledon	Activated charcoal	0.00 ±0.00b	0.00± 0.00a
Nucellus	Activated charcoal	0.00±0.00b	0.00± 0.00a
Testa	Activated charcoal	0.00±0.00b	0.00± 0.00a

The means followed by the same alphabetical letter of the same character and for the same factor are not significantly different (P> 0.05) according to the test for the Least Significant Difference

Table 10. Combined effect of BAP at 0.25 mg/l, 2,4-D and antioxidants polyvinylpyrrolidone (pvp) and activated charcoal on callus formation and survival (mean ± standard errors)

Explants	Antioxidants	Callus (28 days)	Callus (90 days)
Cotyledon	polyvinylpyrrolidone	2.40±0.29a	2.40±0.29a
Nucellus	polyvinylpyrrolidone	0.20±0.00b	0.20±0.00a
Testa	polyvinylpyrrolidone	0.00±0.00b	0.00±0.00a
Cotyledon	Activated charcoal	0.00±0.00b	0.40±0.29a
Nucellus	Activated charcoal	0.60±0.29b	0.60±0.29a
Testa	Activated charcoal	0.00±0.00b	0.00±0.00a

The means followed by the same alphabetical letter of the same character and for the same factor are not significantly different (P> 0.05) according to the test for the Least Significant Difference

4. DISCUSSION

The results of this study showed that nucellus and cotyledons were the best explants for somatic embryogenesis in cashew. Cotyledons produced more calli than nucellus. However, the calli of nucellus were embryogenic, while those of cotyledons were not all embryogenic. Rangaswamy [14] has highlighted the potential of nucellus as an explant for in vitro research of woody plants. Nucellus tissue is of maternal origin, so the desirable traits of the parent are

retained in the progeny derived from this tissue. Furthermore, due to the lack of vascular connection with the parent plant, the nucellar is regarded pathogen-free, and therefore plants of nucellar origin are disease-free. However, it has been observed that the hormone level of the whole kernel poorly reflects the levels in the immature embryos since the endosperm constitutes the majority of the dry matter of the kernel and, as demonstrated by Jiménez and Bangerth [15,16], the hormone levels in the endosperm can vary considerably from those in the immature embryos. Some authors believe that the level of endogenous phytohormones is very low in immature nut explants [17], which would be related to the days of incubation needed to trigger callogenesis. Therefore, in sum, callus formation with nucellus can be observed after 90 days of initial culture. The expression of morphogenetic competence *in vitro* is complex and influenced by physiological and genetic factors. Thus, the effect of explant type and concentration of BAP and 2,4-D was significant on callus formation, in line with other work on *Triticum durum* (durum wheat). Work on mango embryogenesis showed that the combination of 1 mg/l 2,4-D and 0.25 mg/l BAP was the best for callus formation [8]. Culturing explants in 2,4-D-containing media, which is the traditional induction method for many species, has been shown to enhance endogenous auxin levels in the explants [18]. It has also been discovered that polar auxin transport is required for the formation of bilateral symmetry in dicotyledonous plants during somatic embryogenesis [19].Once the stimulus for further development of somatic embryos has been delivered (i.e., by reducing or removing 2,4-D from the culture medium), these levels must be reduced to allow the establishment of the auxin polar gradient. The gradient cannot be created and somatic embryogenesis cannot be expressed if the levels are excessively low or high, or if they do not decrease following the induction treatment. In all species reported in the literature where embryogenic and non-embryogenic callus lines could be obtained in the presence of 2,4-D, it was observed that embryogenic callus contained higher levels of free IAA than non-embryogenic callus. These higher levels may be important in the establishment of polar auxin transport, which is postulated to be critical in the development of somatic embryogenesis. In Arabidopsis, high auxin levels have been reported in cotyledon primordia and cotyledons [20], consistent with the embryogenic competence displayed by the cotyledonary parts of zygotic embryos [21,22]. Sucrose concentration was found to directly influence the uptake of BAP by sunflower explants, and soon afterwards, the levels of endogenous auxins and cytokinins were altered, triggering an organogenic or embryogenic response [23]. The majority of woody plants and some herbaceous species grown in vitro show a browning of the medium. If this browning was extreme, the explants would turn brown to black, necrose and eventually die [24]. In this study, PVP was found to be almost effective in controlling browning, especially in the cotyledons. This could be due to the specificity of these chemicals for certain plants and species. The specificity of PVP in controlling browning has also been reported by Vaugh and Duke [25]. The addition of activated charcoal to liquid and semi-solid media is a recognized practice and its influence on growth and development can be attributed mainly to the adsorption of inhibitory substances in the culture medium [26-30], drastically decreasing phenolic oxidation or accumulation of brown exudate [31-33], changing the pH of the medium to an optimal level for morphogenesis [34] and establishing a darkened environment in the medium and thus simulating soil conditions [35].

Table 11. Combined effect of BAP at 0.5mg/l, 2,4-D; polyvinylpyrrolidone (pvp) and activated charcoal on callus formation and survival (mean ± standard errors)

Explants	Antioxidants	Callus (28 days)	Callus (90 days)
Cotyledon	polyvinylpyrrolidone	0.80±0.25a	3.00±0.23a
Nucellus	polyvinylpyrrolidone	0.40±0.25a	0.00±0.00b
Testa	polyvinylpyrrolidone	0.00±0.00b	0.00±0.00b
Cotyledon	Activated charcoal	0.00±0.00b	0.00±0.00b
Nucellus	Activated charcoal	0.00±0.00b	0.20±0.00b
Testa	Activated charcoal	0.00±0.00b	0.00±0.00b

The means followed by the same alphabetical letter of the same character and for the same factor are not significantly different (P> 0.05) according to the test for the Least Significant Difference

5. CONCLUSION

This study shows that for obtaining viable and embryogenic callus, the combination of BAP at 0.25 mg/L and 0.8 mg/L of 2,4-D is the best and this in the presence of PVP as an antioxidant. The nucellus respond better in the presence of activated charcoal. A protocol of pro-embryonic calli formation from the nucellus, and cotyledons has been established. This protocol has as its basal medium: B5 macroelements, MS microelements, vitamins MS and 400mg/l of L-Glutamine, 500 mg/l of casein hydrolysate, 2.5g/L of Polyvinylpyrrolidone (PVP), 6% of Sucrose. The BAP and 2,4-D growth regulators are associated alone or in combination. As for the type of explant, the testa does not significantly induce the formation ($p>0.05$) of callus compared to nucellus (12.06%) and cotyledon (20.7%) that induce callus.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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