Original Research Article

Influences of 6-Benzylaminopurine, activated charcoal and polyvinylpyrrolidone on the production of embryogenic calli in cashew (*Anacardium occidentale* L.) through cotyledon, nucellus and testa

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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 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

1. INTRODUCTION

Cashew (*Anacardium occidentale* L.) is an economically important horticultural crop of several tropical regions like Asia, Africa, India and Latin America. The cashew tree (*A. occidentale* L.) is highly valued for its edible nuts and shell. It has attracted interest from conventional plant breeders and biotechnology programs with the goal of improving productivity. The growing demand of cashew kernels has led to increased cultivation of the crop. However, current propagation methods have become a limiting factor in supplying adequate planting material [1]. Conventional vegetative propagation methods viz. air layering, grafting or cutting are not rapid to meet the need of elite varieties in time. The commercial technology for plant massive multiplication is primarily based on micropropagation. Micropropagation in particular somatic embryogenesis protocols are aimed at the rapid multiplication of plantlets true-to-type to the original material [2].

Although successes in somatic embryogenesis have been documented for some highly productive forest or fruit species, there are still some obstacles to the implementation of somatic embryogenesis at the operational scale in forestry and agroforestry [3]. Among other constraints of cashew micropropagation, the browning of the explants and the type of growth regulator are the primary factors to be controlled in the induction of somatic embryogenesis [4]. In *in vitro* culture, the cashew tree like the other Anacardiaceae is highly 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 element in 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 the cashew tree, have shown the improvement in embryo production is observed when the nitrogen concentration in the medium, in particular the ammonium salts are weak and 2,4-D replaced by BAP at low concentration (0.25 mg/l).

In addition, the cashew tree is rich in phenolic compounds. Mantell *et al.* [5] report that secondary metabolites are released into the phloem vessels of all organs of the cashew tree. In fact, the injuries caused during the cutting of explants promote the oxidation of these phenolic compounds, the exudation of which leads to browning and then necrosis of the calli. Thus, a low level of calli is obtained at the end of the induction. Establishing an effective protocol for controlling browning is therefore a challenge. Culturing explants with antioxidants such as 0.3% Polyvinylpyrrolidone (PVP) [9] associated with frequent subcultures and dark incubation [10], addition of activated charcoal to the basal medium [11], minimize the effect of phenolic exudations and consequently, the necrosis of the explants. Thus, this work aims to evaluate the effect of BAP and 2,4-D in association with PVP or activated charcoal in the formation of pro-embryogenic calli in order to obtain somatic embryos capable of germinating and regenerating *in vitro* the whole plant of *Anacardium occidentale.*

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. The numbers of explants showing callus and pro-embryonic calli (PEC) initiation as well as the number of days taken for callusing and PEC formation were recorded after 28 and 90 days.

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.

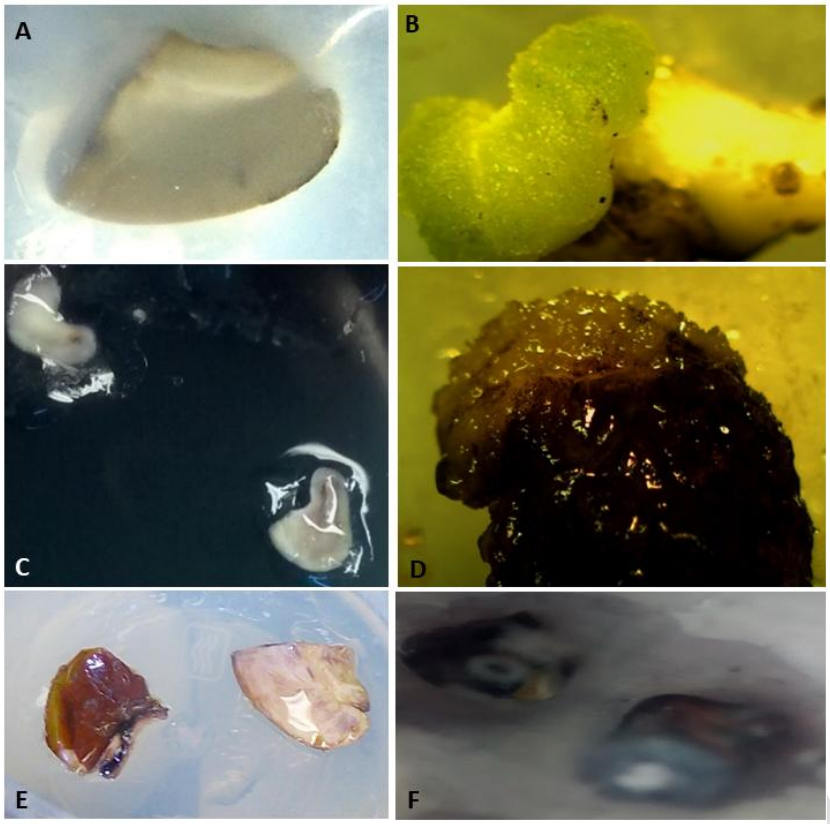
2.3. Statistical analysis

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].

3. RESULTS

3.1. Morphological Response

Morphological changes such as swelling, color change were noticed already at the first week of culture. Regardless of the treatment, 98% of the explants had morphological responses. After the first week of cultivation, the cotyledons have greened and have tripled or even quadrupled in size after three weeks of culture. The explants reacted differently and according to the composition of the medium. At the cotyledons, the calli were observed on the edge and at the outer side. In the nucellus, calli were observed on the surface above the medium. The testa has given few calli and this on the outer layer. The testa (Fig. E) quickly turns brown and dies (Fig. F). Cotyledons and nucellus are the best explants to obtain the callus. Nucellar callus are brown (Fig. D), while cotyledonary calli are snow white, translucent, white crystals or green (Fig. B).



A: Cotyledon explant; B: Calli from Cotyledon; C: Nucellar explant; D: Calli from Nucellar; E: Testa explant; F: Necrosis of testa explant

Figure 1: Morphological response of different explants on the medium

3.2. Callus formation

Analysis of three-factor variances considering media, antioxidants and explants on callus formation after four weeks of cultivation reveals (Table 3) no significant difference ($p>0.05$) between plant growth regulators and antioxidants with respect to callus formation. However, the interaction between hormonal composition and antioxidants significantly influences ($p < 0.05$) the induction of callus. The analysis of variance performed on the data collected four weeks after initial culture of the explants revealed (Table 2) no significant difference ($p>0.05$) between the growth regulators and the antioxidants with regard to callus formation. However, the interaction between hormone composition and antioxidants significantly ($p<0.05$) influences callus induction. Table 3 shows the results of the smallest significant difference test. From this table it can be seen that the testa does not significantly ($p>0.05$) induce callus formation compared to the nucellus and cotyledon, which do induce callus and even of medium size. Activated carbon appears to be the antioxidant that significantly ($p < 0.05$) favours callus formation in the medium where there is only 2,4-D at 0.8 mg/l (C0) and the medium where BAP is at 0.25 mg/l and 2,4-D is absent (C1). The medium (C1) is the least favourable for callus formation. However, the media C0 where BAP is zero (0mg/l) and 2,4-D 0.8mg/l; then C3 where BAP is low (0.25mg/l) and 2,4-D 0.8mg/l produce significantly ($p < 0.05$) more callus (0.47 ±0.00 on average) according to the test of the Least Significant Difference. In general, the C0, C4 and C3 media were the best for obtaining average callus with 46%, 37% and 23% of

average callus respectively. It was also noted that activated charcoal gave more callus on media C0 and C1. Generally, we note that the C0 media where the BAP is zero (0mg/l) and the 2,4-D 0.8mg/l; then C3 where the BAP is low (0.25mg/l) and the 2,4-D 0.8mg/l produce 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	0.85ns	0.69^{ns}	1.03^{ns}	2.58^{ns}	0.12^{ns}
Antioxidants	1	0.09ns	0.55^{ns}	0.05^{ns}	0.90^{ns}	0.20^{ns}
Explants	2	3.06ns	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	0.64ns	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*

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±0,00**b**	0,00±0,00 **a**	1,00±0,00**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**b**	0,00±0,00**a**	0,00±0,00**a**
		Cotyledon	0,00±0,00**b**	0,00±0,00 **a**	1,00±0,00**a**	0,00±0,00a	0,00±0,00**a**
		Mean	0,20±0,20**B**	0,00±0,00 **A**	0,80±0,20**A**	0,00±0,00**A**	0,00±0,00**A**
	PVP	Nucellus	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**
		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	0,50±0,28**a**	0,00±0,00 **a**	0,50±0,28**a**	0,00±0,00**a**	0,00±0,00**a**
		Mean	0,750±0,16**A**	0,00±0,00 **A**	0,25±0,16**B**	0,00±0,00**A**	0,00±0,00**A**
	Mean		0,53±0,00**Y**	0,00±0,00 **X**	0,46±0,00**X**	0,00±0,00**X**	0,00±0,00**X**
BAP 0,25 mg/l and 2,4-D 0mg/l (C1)	Activated charcoal	Nucellus	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**
		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	0,00±0,00**a**	0,00±0,00 **a**	1,00±0,00**a**	0,00±0,00**a**	0,00±0,00**a**
		Mean	0,66±0,33**B**	0,00±0,00 **A**	0,33±0,33**A**	0,00±0,00**A**	0,00±0,00**A**
	PVP	Nucellus	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**
		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	1,00±0,00**A**	0,00±0,00 **A**	0,00±0,00**B**	0,00±0,00**A**	0,00±0,00**A**
	Mean		0,90±0,00**X**	0,00±0,00 **X**	0,10±0,00**Y**	0,00±0,00**X**	0,00±0,00**X**

BAP 0,5mg/l and 2,4-D 0mg/l (C2)	Activated charcoal	Nucellus	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**
		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	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**
	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±0,15**A**	0,00±0,00 **A**	0,20±0,13**A**	0,00±0,00**A**	0,10±0,10**A**
	Mean		0,78±0,00**X,Y**	0,00±0,00 **X**	0,14±0,00**X,Y**	0,00±0,00**X**	0,07±0,00**X**
BAP 0,25mg/l and 2,4-D 0,8mg/l (C3)	Activated charcoal	Nucellus	0,00±0,00**b**	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±0,28**A**	0,25±0,25 **A**	0,25±0,25**A**	0,00±0,00**A**	0,00±0,00**A**
	PVP	Nucellus	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**
		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	0,20±0,20 **a**	0,00±0,00 **a**	0,40±0,24**a**	0,00±0,00**a**	0,40±0,24**a**
		Mean	0,55±0,17**A**	0,00±0,00 **A**	0,22±0,14**A**	0,00±0,00**A**	0,22±0,14**A**
	Mean		0,53±0,00**Y**	0,07±0,00 **X**	0,23±0,00**X,Y**	0,00±0,00**X**	0,15±0,00**X**
BAP 0,5mg/l and 2,4-	Activated charcoal	Nucellus	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**

D 0,8mg/l (C4)		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	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**
	PVP	Nucellus	0,00±0,00**a**	0,00±0,00**a**	1,00±0,00**a**	0,00±0,00**a**	0,00±0,00**a**
		Testa	0,00±0,00**a**	0,00±0,00**a**	0,00±0,00**a**	0,00±0,00**a**	0,00±0,00**a**
		Cotyledon	0,33±0,33**a**	0,00±0,00**a**	0,66±0,33**a**	0,00±0,00**a**	0,00±0,00**a**
		Mean	0,25±0,25**A**	0,00±0,00**A**	0,75±0,25**A**	0,00±0,00**A**	0,00±0,00**A**
	Mean		0,62±0,00**X,Y**	0,00±0,00**X**	0,37±0,00**X,Y**	0,00±0,00**X**	0,00±0,00**X**

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

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±0,00**a**	0,00±0,00**a**	0,00±0,00**a**	0,00±0,00**a**	0,00±0,00**a**
	PVP	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	1,00±0,00**A**	0,00±0,00**B**	0,00±0,00**A**	0,00±0,00**A**	0,00±0,00**B**
BAP 0,25mg/l and 2,4-D 0mg/l (C1)	Activated charcoal	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**
	PVP	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	1,00±0,00**A**	0,00±0,00**B**	0,00±0,00**A**	0,00±0,00**A**	0,00±0,00**B**
BAP 0,5mg/l and 2,4-D 0mg/l (C2)	Activated charcoal	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**
	PVP	0,92±0,07**a**	0,00±0,00**a**	0,00±0,00**a**	0,07±0,07**a**	0,00±0,00**a**
	Mean	0,94±0,05**A**	0,00±0,00**B**	0,00±0,00**A**	0,05±0,05**A**	0,00±0,00**B**
BAP 0,25mg/l and 2,4-D 0,8mg/l (C3)	Activated charcoal	0,91±0,08**a**	0,08±0,08**b**	0,00±0,00**a**	0,00±0,00**a**	0,00±0,00**a**
	PVP	0,23±0,07**b**	0,40±0,09**a**	0,16±0,06**a**	0,20±0,07**a**	0,00±0,00**a**
	Mean	0,42±0,07**B**	0,30±0,07**A**	0,11±0,05**A**	0,14±0,05**A**	0,00±0,00**B**
BAP 0,5mg/l and 2,4-D	Activated charcoal	0,88±0,11**a**	0,00±0,00**a**	0,00±0,00**a**	0,11±0,11**a**	0,00±0,00**a**

0,8mg/l (C4)	PVP	0,50±0,13**a**	0,07±0,07**a**	0,00±0,00**a**	0,21±0,11**a**	0,21±0,11**a**
	Mean	0,65±0,10**B**	0,04±0,04**B**	0,00±0,00**A**	0,17±0,08**A**	0,13±0,07**A**

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.

3.2.1. 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 is noticed that, the existence of both growth regulators in the medium is important for callogenesis in cashew. Medium C3: 0.25 mg/l of BAP and 0.8 mg/l of 2,4-D appear to be the best concentration to optimize the production of calli 58%.

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±0.00**a**	1.00±0.00**a**	0.94±0.05**a**	0.42±0.07**b**	0.65±0.10**b**
Slight callus	0.00±0.00**b**	0.00±0.00**b**	0.00±0.00**a**	0.30±0.07**a**	0.04±0.04**b**
Moderate callus	0.00±0.00**a**	0.00±0.00**a**	0.00±0.00**a**	0.11±0.05**a**	0.00±0.00**a**
Profuse callus	0.00±0.00**a**	0.00±0.00**a**	0.05±0.05**a**	0.14±0.05**a**	0.17±0.08**a**
highly profuse callus	0.00±0.00**b**	0.00±0.00**b**	0.00±0.00**b**	0.00±0.00**b**	0.13±0.07**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

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 calli from cotyledons with both activated charcoal and polyvinylpyrrolidone. On medium with activated charcoal, the nucellus also form callus. But the formation of calli is observed 28 days after the initial culture and the calli did not last over time. Activated charcoal appears to be the antioxidant significantly promoting ($p < 0.05$) the formation of calli on the medium where there is only 2,4-D at 0.8 mg/l (C0).

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±0.32**a**	0.00±0.00**a**
testa	Activated charcoal	0.00±0.32**b**	0.00±0.00**a**
Cotyledon	Activated charcoal	0.50±0.36**a**	0.00±0.00**a**
Nucellus	polyvinylpyrrolidone	0.00±0.32**b**	0.00±0.00**a**
testa	polyvinylpyrrolidone	0.00±0.32**b**	0.00±0.00**a**
Cotyledon	polyvinylpyrrolidone	1.00±0.36**a**	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

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

Only cotyledons make it possible to obtain calli as much with activated charcoal as polyvinylpyrrolidone with BAP at 0.25 mg/l. These calli appear after 28 days of initial culture but do not survive.

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±0.283**a**	0.00±0.00 **a**
Nucellus	polyvinylpyrrolidone	0.000±0.00**a**	0.00±0.00 **a**
Testa	polyvinylpyrrolidone	0.000±0.00**a**	0.00±0.00 **a**
Cotyledon	Activated charcoal	0.800±0.00**a**	0.00±0.00 **a**
Nucellus	Activated charcoal	0.000±0.00**a**	0.00±0.00 **a**
Testa	Activated charcoal	0.000±0.00**a**	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

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

BAP at 0.5 mg/l produces callus from nucellus and cotyledons but only with polyvinylpyrrolidone as antioxidant (Table 9). Callus from cotyledons lasted up to 90 days, but the rate decreased by half. On the other hand, with activated charcoal no callus production was noted for any explant.

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±0.36**a**	0.60 ± 0.24**a**
Nucellus	polyvinylpyrrolidone	0.40±0.36**a**	0.00± 0.00**a**
Testa	polyvinylpyrrolidone	0.00±0.00**b**	0.00± 0.00**a**
Cotyledon	Activated charcoal	0.00 ±0.00**b**	0.00± 0.00**a**
Nucellus	Activated charcoal	0.00±0.00**b**	0.00± 0.00**a**
Testa	Activated charcoal	0.00±0.00**b**	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

3.2.5. Combined effect of BAP at 0.25mg/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 shows that the combination of BAP at 0.25mg/l and 2,4-D at 0.8mg/l resulted in 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 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.

Table 10: Combined effect of BAP at 0.25mg/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±0.29**a**	2.40±0.29**a**
Nucellus	polyvinylpyrrolidone	0.20±0.00**b**	0.20±0.00**a**
Testa	polyvinylpyrrolidone	0.00±0.00**b**	0.00±0.00**a**
Cotyledon	Activated charcoal	0.00±0.00**b**	0.40±0.29**a**
Nucellus	Activated charcoal	0.60±0.29**b**	0.60±0.29**a**
Testa	Activated charcoal	0.00±0.00**b**	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

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

Table 11 shows that the presence of 0.5mg/l of BAP, 0.8 mg/l of 2,4-D and 2.5 g/l of polyvinylpyrrolidone increases the amount of callus with cotyledons. The combined presence of BAP and 2,4-D growth regulators increases the amount of callus formed. In addition, with activated charcoal, whatever the explant, no callus production is noted except for the nucelli which produce after 90 days 0.20±0.00 of callus.

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±0.25**a**	3.00±0.23**a**
Nucellus	polyvinylpyrrolidone	0.40±0.25**a**	0.00±0.00**b**
Testa	polyvinylpyrrolidone	0.00±0.00**b**	0.00±0.00**b**
Cotyledon	Activated charcoal	0.00±0.00**b**	0.00±0.00**b**
Nucellus	Activated charcoal	0.00±0.00**b**	0.20±0.00**b**
Testa	Activated charcoal	0.00±0.00**b**	0.00±0.00**b**

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 show that nucellus and cotyledons are the best explants for somatic embryogenesis in cashew. Cotyledons produce more calli than nucellus. However, nucellar calli are embryogenic, whereas not all cotyledons are. Potential of nucellus as an explant for *in vitro* studies involving woody plants has been emphasized by Rangaswamy [14]. The nucellar tissue is maternal in origin, hence desirable traits of the parent are retained in the offspring arising from this tissue. Also, due to lack of vascular connection with the parent plant, nucellus is considered free of pathogens, and therefore the plants having a nucellar origin would be disease-free. However, it has been observed that the whole kernel hormone level poorly reflects the levels in the immature embryos since the endosperm constitutes the majority of kernel dry matter, and, as was demonstrated by Jiménez and Bangerth [15,16], hormone levels in the endosperm might vary greatly in relation to those of the immature embryos. Some authors believe that the level of endogenous phytohormones is very low in immature nut explants [17], which is related to the incubation days required to trigger callogenesis. This is why, in sum, callus formation with nucellus can be seen 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 the type of explant and the concentration of BAP and 2,4-D was significant on the formation of callus in accordance with other work done on *Triticum durum* (durum wheat). Mango work in embryogenesis showed that the combination of 1 mg/l of 2,4-D and 0.25 mg/l of BAP was shown to be the best in callus formation [8]. It has been reported that the culture of explants in medium

containing 2,4-D, the classic induction treatment for many species, increases the endogenous auxin levels in the explants [18]. It has also been observed that polar transport of auxin is essential for the establishment of bilateral symmetry during embryogenesis in dicotyledonous somatic [19]. Once the stimulus for the further development of the somatic embryos is given (i.e., through reduction or removal of 2,4-D from the culture medium), those levels must be reduced, to allow the establishment of the polar auxin gradient. If the levels are extremely low or high, or if they do not diminish after the induction treatment, the gradient cannot be formed, and thus somatic embryogenesis cannot be expressed. In all species reported in the literature in which embryogenic and non-embryogenic callus lines could be obtained in the presence of 2,4-D, it was observed that the embryogenic calli contained higher levels of free IAA than the nonembryogenic ones. These higher levels could be important in the establishment of polar auxin transport, which is postulated to be determinant in somatic embryogenesis development. In Arabidopsis high level of auxins has been indicated in cotyledon primordia and cotyledons [20], which correlates with embryogenic competence displayed by cotyledonary parts of zygotic embryos [21,22]. Sucrose concentration has been found to directly influence the uptake of BAP into the sunflower explants, and shortly later endogenous auxins and cytokinin level was modified which in turn triggered organogenic or embryogenic response [23]. The majority of woody plants and some herbaceous species under *in vitro* culture shows browning of medium. If this browning was so extreme, the explants turn its colour brown to black and become necrotic and finally lead to die [24]. In this study, PVP was found almost effective in browning control especially for the cotyledons. This could be due to the specificity of these chemicals to certain plant and species. The specificity of PVP in browning control was also reported by Vaugh and Duke [25]. The addition of Activated Charcoal to both liquid and semi-solid media is a recognized practice and its influence in growth and development may be attributed mainly to the adsorption of inhibitory substances in the culture medium [26-30], drastic decrease in the phenolic oxidation or brown exudate accumulation [31-33], alteration of medium pH to an optimum level for morphogenesis [34] and establishment of a darkened environment in medium and hence simulate soil conditions [35].

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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DEFINITIONS, ACRONYMS, 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