Scientific Research Publishing

Advances in Bioscience and Biotechnology, 2022, 13, 388-400
https://www.scirp.org/journal/abb
ISSN Online: 2156-8502
ISSN Print: 2156-8456

Buds Reactivity and Factors Promoting Shoots Proliferation and Rooting of Cashew Seedlings Using *in Vitro* Tissue Culture Process

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How to cite this paper: Akakpo, B.S.A., Agbidinoukoun, A., Ahanhanzo, C., Cacai, G.H.T., Badou, B.T., Houedjissin, S. and Dossou, J. (2022) Buds Reactivity and Factors Promoting Shoots Proliferation and Rooting of Cashew Seedlings Using *in Vitro* Tissue Culture Process. *Advances in Bioscience and Biotechnology*, **13**, 388-400.
https://doi.org/10.4236/abb.2022.139025

Received: June 11, 2022
Accepted: September 17, 2022
Published: September 20, 2022

Abstract

Tissue culture techniques are widely used for the mass propagation of many species. In cashew *in vitro* propagation, some protocols need to be established at this end. The present work was carried out to evaluate the conditions for *in vitro* regeneration of cashew seedlings from micropropagation by organogenesis on Benin genotypes. Nodal explants from one-month-old cashew seedlings in the greenhouse and cotyledonary nodes from *in vitro* germination were used for this purpose. BAP and kinetin were evaluated alone at 2.2 mg/L and then the combination of 2.2 mg/L BAP + 0.2 mg/L IBA was also evaluated. The response of axillary bud proliferation on explants was obtained with both cotyledonary nodes and axillary buds from different combinations of growth regulators. However, the best responses were recorded with cotyledonary nodes. When 2.2 mg/L BAP was used, 80% of the explants responded with numerous proliferation (5 to 8) buds (5.75 ± 0.12) with good shoot length (6.73 ± 0.3 cm) on MS medium containing 150 mL coconut water. Rooting was observed with the combination of NAA (2.5 mg/l) + IBA (2.5 mg/l) on ½ MS containing 40 g/l sucrose.

Keywords

Micropropagation, Cashew Elite Genotypes, Buds, *In Vitro* Regeneration, Organogenesis

1. Introduction

Anacardium occidentale is a strategic product for the whole world. Cashew nuts are an export product that is increasingly in demand on international markets. Cashew nuts offer economic opportunities throughout the value chains. Through local processing, it offers considerable potential for industrial development in producing countries. Globally, cashew trade exceeds US$2 billion and demand is increasing. Of the total world supply, 110,000 t are traded on international markets: India (60%) and Brazil (31%) are the main exporters [1]. In areas with very low rainfall, the young leaves are edible. The wood is highly valued [2] as it is quite hard with a high density of about 500 kg/m^3 [3], and is used as timber, firewood and for charcoal production [4]. The bark and leaves are used in folk medicine [5]. Cashew leaves and bark have fungicidal and bactericidal properties. Other authors have shown that the balsam (product of the shell) has interesting biological properties on certain pests [6] [7]. It is becoming a sector that brings together all energies. But as a commodity chain, if Africa were only to play the role of producing the raw material, it would not be developed. Recently, Masawe [8] established the first polyclonal field in Tanzania, the second in Mozambique and the third in Benin. The development of polyclonal seeds is an asset. However, this technique also needs modern plant biotechnology techniques. Micropropagation can be useful for producing clonal rootstocks and for multiplying breeders' stock more rapidly. Several studies indicate that root initiation is very low in cashew grafting due to the difficulty of micrografted shoots to fully recover their rhizogenesis capacity after a single grafting cycle. Previous results have repeatedly shown that reversion can take time and that successful rejuvenation may require 4 - 6 successive grafts before signs of juvenility are detectable [9]. Therefore, a second plant biotechnology technique: micropropagation by organogenesis is used for seed multiplication. This increases the supply of grafts (potential use of grafted plants as sources of "reinvigorated" bud material for rapid clonal micropropagation) and bypasses the difficulty of rooting by grafting. Biotechnology through *in vitro* culture will allow massive vegetative production of the various elite genotypes to have a large number of copies of each genotype to constitute high-yield orchards. Moreover, with a kernel yield (KOR value) between 47 and 49 pounds (lbs), Benin nuts have a worldwide reputation for their exceptional quality and taste [10]. However, cashew like other Anacardiaceae, presents many constraints of micropropagation. One of the main constraints of *in vitro* culture of cashew is the high production of secondary metabolites as a result of organ harvesting injuries [11]. Indeed, oxidation of these compounds causes browning and necrosis of organs in the culture medium. Similarly, the high level of disinfection required for decontamination makes it difficult for field-collected explants to survive [12] [13]. These authors recorded survival rates of 3% and 25% for shoot tips and nodal explants of cultivated plants. Most explants that survived after disinfection became brown or necrotic after 20 days of culture [14] [15]. Often, micropropagation from mature tree ex-

plants is affected by excessive contamination. In many cases, micropropagation from mature tree explants is affected by excessive contamination, phenolic exudation, slow growth, difficulties in elongation and rooting of micro-pods [8]. Several authors [16] [17] [18] have reported that explants excised from *in vitro* germinated seedlings are the most suitable for micropropagation of elite cashew trees. Although protocols for *in vitro* regeneration of cashew exist, in Benin no studies on *in vitro* culture of local cashew varieties have been initiated. The objective of this work was to evaluate the bud reactivity and factors favoring *in vitro* multiplication and rooting of cashew seedlings produced in Benin in order to produce rejuvenated elite shoots.

2. Materials and Methods

2.1. Material

The plant material consisted of shoots from micrografting and seedlings from germinating nuts of successful cashew trees.

2.2. Methods

2.2.1. Cashew Shoot Initiation

- **Preparation, disinfection, and culturing of explants**

To prepare explants from 4-week-old vitroplants, under a laminar flow hood, the vitroplants were carefully removed from the jars and placed in a large sterile petri dish (14.5 cm diameter). Using forceps and a scalpel with a sterile blade, the apex and cotyledonary node (2.5 - 3.0 cm long) were removed. The cotyledonary nodal explant with and without broad cotyledons is obtained by discarding the root portion and retaining about 0.5 - 1.0 cm of the hypocotyl and epicotyl portion on each side of the cotyledonary node junction. Single or double nodal cuttings (1 cm in length) were prepared by segmenting the terminal shoot after defoliation. The explants thus prepared were grown on a shoot initiation medium. On the other hand, explants taken in the greenhouse (cuttings and apices 1.0 to 1.5 cm long) after sterilization were grown in tubes (25 × 250 mm) for four weeks. Several subcultures were performed. First, the subcultures were done at weekly intervals until the third week, and then at an interval of three weeks. The apices become dark and necrotic if not frequently cultured.

- **Preparation of the culture medium**

The shoot initiation medium consisted of a modified medium (three-quarters macroelements, complete microelements, vitamins) of Murashige and Skoog [19] supplemented with 3% sucrose, 0.2% activated carbon (AC) and solidified with 2.25 g/L phytagel (Sigma). The pH of the medium was adjusted to 5.8 before autoclaving at 121˚C for 20 min under a pressure of 1 bar. The autoclaved medium was dispensed into 100 mL test tubes with 15 mL of medium per tube.

- **Growing conditions**

Cultures were incubated in a culture room at 25˚C ± 2˚C, under a 16 h photoperiod. Initially, for 3 weeks, low light intensity (16 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was pro-

vided. Subsequently, the light intensity was increased (45 - 55 µmol·m^{-2}·s^{-1}) and supplied from fluorescent lamps (Philips, 40 W).

2.2.2. Axillary Bud Proliferation

Axillary bud multiplication was carried out on a multiplication medium consisting of modified MS (3/4) with 3% sucrose and 25 mg/L cysteine. BAP and kinetin were assessed at 2.2 mg/L alone or in combination in the presence of 0.2 mg/L AIB. The culture medium was solidified with 8 g/L Agar. After four weeks, the cultures were transferred to MS/2 medium without growth regulators.

The number of axillary buds and shoots formed were assessed at four and ten weeks by counting.

2.2.3. Shoot Bud Elongation

Axillary buds induced on the propagation medium were grown on MS/2 medium without growth regulator supplemented with 0.2% activated charcoal and 400 mg/L glutamine, for leafy shoot elongation. The inoculated cultures were kept at an incubation temperature of 25˚C ± 2˚C under white fuorescent tube lights for 16 h day. The effect of coconut water (150 mL) was tested. Multiple shoots with proliferating buds were divided into two separate clusters and grown at an interval of three weeks. At each transplanting, long shoots were harvested and the remaining buds were put back on the same medium for elongation. After ten weeks the length of the shoots was assessed by measuring with graph paper.

2.2.4. Root Induction

The resulting long (>2 cm) microgrowths were rooted on an MS/2 medium with 25 mg/L cystene and 4% sucrose. NAA and IBA were used in combination (2.5 mg/L each). The cultures were incubated in light. As soon as the root initials appeared, they were transferred to liquid MS/2 medium without a growth regulator containing 1 g/L activated carbon on filter paper. The pH of the medium was adjusted to 5.8 ± 0.1 before autoclaving.

2.2.5. Parameters Assessed

For axillary bud proliferation, the number of axillary buds and shoots formed were assessed at four and ten weeks. For elongation, shoot length was assessed after ten weeks by measuring with graph paper. The number of roots formed was assessed 15 weeks after initiation.

2.2.6. Statistical Analysis

The number of buds, number of shoots, and number of roots were subjected to a three-factor ANOVA (Media, Explant Type, Time). To obtain normal distributions (ANOVA hypothesis), the count data (number of buds, number of shoots, number of roots) were transformed to log10 (n) [20], where n was the real value. Shoot elongation was also subjected to a three-factor ANOVA (media, explant type, time). These analyses were performed using the PROC GLM procedure of the SAS software (Statistical Analysis System Version 9.2). The Student New-

man-Keuls test was used for the separation of means considering a probability level of 5% [21].

3. Results

3.1. Assessment of the Survival Rate of Explants during Proliferation

The survival rate varied with the type of explants and the media. The high survival rate (90%) was recorded by cotyledonary nodes from *in vitro* germination cultured on the medium M3 containing both of the two cytokinins tested (2.2 mg/L BAP + 0.2 mg/L IBA) (**Table 1**).

Shoots on medium without growth regulators started to grow within three weeks with swelling and greening of the axillary buds (**Figures 1(A)-(D)**).

Table 1. Survival rate of explants initiated to micropropagation.

Media	Explant	Period (week after initiation)	Number of survival	Survival rate (%)
M0 (control)	Nodes from the greenhouse	0	10	
		4	7	70
		10	5	50
	Cotyledonary nodes from *in vitro* germination	0	10	
		4	7	70
		10	5	50
M1 (2.2 mg/L BAP)	Nodes from the greenhouse	0	10	
		4	8	80
		10	8	80
	Cotyledonary nodes from *in vitro* germination	0	10	
		4	8	80
		10	8	80
M2 (2.2 mg/L Kin)	Nodes from the greenhouse	0	10	
		4	8	80
		10	7	70
	Cotyledonary nodes from *in vitro* germination	0	10	
		4	8	80
		10	8	80
M3 (2.2 mg/L BAP + 0.2 mg/L IBA)	Nodes from the greenhouse	0	10	
		4	9	90
		10	8	80
	Cotyledonary nodes from *in vitro* germination	0	10	
		4	9	90
		10	9	90

3.2. Assessment of the Effect of Culture Medium, Explant Type, and Time on Proliferation

The results of the analysis of variance of the number of buds, shoots, and roots as a function of growing medium, explant type, and time are given in Table 2. The effect of culture medium, explant type, and time on the number of buds, shoots, and roots (mean ± standard error) was presented in Table 3. The number of buds formed was very significantly ($p < 0.001$) influenced by culture medium, explant type, and time. The number of shoots was not significantly ($p > 0.05$)

Figure 1. Micropropagation of the cashew tree. (A) Cutting apex; (B)-(D): Proliferation of axillary buds; (E), (F): Proliferation of shoots; (G), (H): Shoot elongation.

Table 2. Fischer's value and significance level of the three-factor ANOVA (media, explant type, time) on the number of buds, shoots, and roots.

Source of variation	Degree of freedom	F-Values		
		Number of buds	Number of shoots	Number of roots
Media	3	10.50***	22.08***	2.29ns
Explant type	1	13.98***	1.36ns	2.11ns
Period	1	12.17***	7.05**	2.11ns
Media * Explant type	3	1.57ns	0.84ns	2.29ns
Media * Period	3	0.38ns	1.09ns	2.29ns
Explant type * Period	1	1.53ns	0.10ns	2.11ns
Media* Explant type * Period	3	0.56ns	0.02ns	2.29ns

ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

influenced by explant type but was significantly ($p < 0.05$ and $p < 0.001$) influenced by culture medium and time. Regarding root numbers, none of the factors had a significant effect on root formation.

None of the interactions had a significant effect ($p > 0.05$) on the number of buds, shoots, or roots. From Table 3, it appeared that several axillary buds started to appear and proliferate around the axles of the nodes. The emergence of axillary buds was observed from the first crop. The number of axillary buds formed varied from 1 to 12 with an average of 4-5 buds/crop. The M1 medium where BAP was present at 2.2 mg/l with cotyledonary nodes from *in vitro* germinated seedlings resulted in a higher number of axillary buds (4.56 ± 0.09), shoots (4.19 ± 0.06),

Table 3. Effect of growing medium, explant type, and period on the number of buds, shoots, and roots (mean ± standard error).

Media	Explant type	Period (week after initiation)	Number of buds	Number of shoots	Number of roots
M0 (Control)	Nodes from the greenhouse	4	0.15 ± 0.07a (1.57)	0.00 ± 0.00a (0)	0.00 ± 0.00a (0)
		10	0.00 ± 0.00a (1)	0.00 ± 0.00a (0)	0.00 ± 0.00a (0)
		Mean	0.09 ± 0.05A (1.33)	0.00 ± 0.00A (0)	0.00 ± 0.00A (0)
	Cotyledonary nodes from *in vitro* germination	4	0.2 ± 0.07a (1.86)	0.00 ± 0.00a (0)	0.00 ± 0.00a (0)
		10	0.12 ± 0.07a (1.4)	0.00 ± 0.00a (0.2)	0.00 ± 0.00a (0)
		Mean	0.17 ± 0.05A (1.66)	0.00 ± 0.00A (0.08)	0.00 ± 0.00A (0)
	GENERAL MEAN		0.13 ± 0.04Y (1.5)	0.00 ± 0.00Z (0.04)	0.00 ± 0.00X (0)
M1 (2.2 mg/L BAP)	Nodes from the greenhouse	4	0.27 ± 0.06a (2)	0.31 ± 0.13a (2.37)	0.00 ± 0.00a (0)
		10	0.22 ± 0.09a (1.7)	0.42 ± 0.1a (3.3)	0.00 ± 0.00a (0)
		Mean	0.24 ± 0.06B (1.83)	0.37 ± 0.08A (2.89)	0.00 ± 0.00A (0)
	Cotyledonary nodes from *in vitro* germination	4	0.67 ± 0.12a (5.75)	0.46 ± 0.12a (3.75)	0.00 ± 0.00a (0.25)
		10	0.4 ± 0.14a (3.38)	0.6 ± 0.1a (4.62)	0.08 ± 0.05a (0.63)

Continued

		Mean	0.54 ± 0.09A (4.56)	0.53 ± 0.08A (4.19)	0.03 ± 0.02A (0.44)
	GENERAL MEAN		0.38 ± 0.06X (3.12)	0.45 ± 0.06X (3.5)	0.02 ± 0.01X (0.21)
M2 (2.2 mg/L Kin)	Nodes from the greenhouse	4	0.03 ± 0.03a (0.77)	0.00 ± 0.00a (0.13)	0.00 ± 0.00a (0)
		10	0.03 ± 0.03a (0.78)	0.1 ± 0.07a (1.11)	0.00 ± 0.00a (0)
		Mean	0.04 ± 0.02B (0.94)	0.05 ± 0.03A (0.65)	0.00 ± 0.00A (0)
	Cotyledonary nodes from *in vitro* germination	4	0.3 ± 0.1a (2.38)	0.00 ± 0.00a (0.5)	0.00 ± 0.00a (0)
		10	0.11 ± 0.08a (1.38)	0.11 ± 0.08a (1.38)	0.00 ± 0.00a (0)
		Mean	0.2 ± 0.06A (1.87)	0.06 ± 0.04A (0.94)	0.00 ± 0.00A (0)
	GENERAL MEAN		0.12 ± 0.04Y (1.4)	0.05 ± 0.03Z (0.79)	0.00 ± 0.00X (0)
M3 (2.2 mg/L BAP + 0.2 mg/L IBA)	Nodes from the greenhouse	4	0.21 ± 0.07a (1.78)	0.08 ± 0.08a (0.67)	0.00 ± 0.00a (0)
		10	0.04 ± 0.04a (1)	0.28 ± 0.12a (2.29)	0.00 ± 0.00a (0)
		Mean	0.14 ± 0.05A (1.44)	0.16 ± 0.07A (1.37)	0.00 ± 0.00A (0)
	Cotyledonary nodes from *in vitro* germination	4	0.33 ± 0.08a (2.44)	0.08 ± 0.08a (0.78)	0.00 ± 0.00a (0)
		10	0.08 ± 0.05b (1.13)	0.34 ± 0.09a (2.5)	0.00 ± 0.00a (0)
		Mean	0.21 ± 0.06A (1.82)	0.2 ± 0.07A (1.59)	0.00 ± 0.00A (0)
	GENERAL MEAN		0.17 ± 0.04Y (1.64)	0.18 ± 0.05Y (1.48)	0.00 ± 0.00X (0)

Within a column, means followed by letters of the same characters are not significantly different ($P > 0.05$) according to the Student Newman-Keuls test. The real values in brackets have been transformed by log10(n).

Table 4. Fischer's value and significance level of the three-factor ANOVA (media, explant type, period) on shoot elongation.

Source of variation	Degree of freedom	F-Values Length of shoots (cm)
Media	1	15.38***
Explant type	1	198.02***
Period	1	217.98***
Repetition	14	0.94ns
Media * Explant type	1	00ns
Media * Period	1	15.38***
Explant type * Period	1	0.05ns
Media* Explant type * Period	1	00ns

ns: $p > 0.05$; ***: $p < 0.001$.

and roots (0.44 ± 0.02) already from four weeks.

3.3. Evaluation of the Effect of Environment, Explant Type, and Time on Shoot Elongation

After 10 weeks, shoot elongation was observed at a frequency of 70% - 80%. The analysis of variance (ANOVA) performed on shoot length as a function of culture medium, explant type, and period (Table 4) revealed that the latter (culture medium, explant type, period) as well as the medium * period interaction influenced shoot elongation very significantly ($p < 0.001$).

Analysis of Table 5 showed that the E2 medium with 150 mL coconut water was the best medium with an average of 4.35 ± 0.24 cm. Furthermore, cotyledonary nodes from *in vitro* germination were found to be the best explants ($p < 0.05$) for micropropagation with an average length of 5.37 ± 0.29 cm.

Although the number of roots formed varies (1 - 4/crop) (Figure 2), the majority have one to two prominent roots reaching an average length of four (4) cm. The percentage of rooting varies from 20% - 30%. The reduction in salt concentration and the increase in sucrose concentration favored rooting. Shoots harvested from the cotyledonary nodes showed a higher rooting percentage.

4. Discussion

The present work used nodal explants from one-month-old cashew plants in the greenhouse and cotyledonary nodes from *in vitro* germination. The response of axillary bud proliferation on the explants was obtained with both cotyledonary nodes and axillary buds from different combinations of growth regulators. However, the best responses were recorded with the cotyledonary nodes. The results also revealed that 80% of the explants responded with numerous proliferation (12 - 15) of buds (5.75 ± 0.12) with good shoot length (6.73 ± 0.3 cm) on MS medium containing 150 mL coconut water and 2.2 mg/L BAP. The efficacy

Table 5. Effect of culture medium, explant type, and period on shoot length (mean ± standard error).

Media	Explant type	Temps (semaines après initiation)	Length of shoots (cm)
E1	Nodes from the greenhouse	4	2 ± 0b
		10	3.53 ± 0.26a
		Mean	2.77 ± 0.19B
	Cotyledonary nodes from *in vitro* germination	4	4 ± 0b
		10	5.6 ± 0.27a
		Mean	4.8 ± 0.2A
	GENERAL MEAN		3.78 ± 0.19Y
E2 (MB + 150 mL CW)	Nodes from the greenhouse	4	2 ± 0b
		10	4.67 ± 0.32a
		Mean	3.33 ± 0.29B
	Cotyledonary nodes from *in vitro* germination	4	4 ± 0b
		10	6.73 ± 0.3a
		Mean	5.37 ± 0.29A
	GENERAL MEAN		4.35 ± 0.24X

Means followed by the same letter and for the same factor are not significantly different ($p > 0.05$) according to the Student Newman-Keuls test.

Figure 2. Elongation of neoformed shoots.

of BAP over kinetin for shoot proliferation of *Morinda citrifolia* has been reported [22]. The higher number of buds obtained with cotyledonary nodes could be due to the larger meristematic area at the cotyledonary node, in contrast to nodal explants where this area is smaller. A higher potential for bud induction (12 buds) by cotyledonary nodes compared to other explants on MS medium was reported by [12] on cashew tissue culture and regeneration of *Dacryodes edulis [23]*. Similarly, in 1995, [14] obtained nine buds per explant from cotyledonary nodes of *Anacardium occidentale.* Large shoots were induced from the

cotyledonary nodes, possibly due to the presence of nutrient reserves, as the cotyledons are nutrient reserve structures for the subsequent growth of the seedling, while the roots allow the explant to absorb nutrients from the environment to provide energy for the growing seedlings.

Furthermore, to optimize rooting responses, the combination of NAA (2.5 mg/l) + IBA (2.5 mg/l) was used on 1/2 MS containing 40 g/l sucrose. Halving the mineral element concentrations of the MS medium and increasing the sucrose concentration are essential in cashew rhizogenesis. Indeed, the reduction of the mineral concentration in the medium leads to a decrease in the nutritive resources of this medium. On the other hand, sucrose is involved in growth equilibrium and the localization of mitoses [24]. Indeed, these authors stated that high concentrations of sucrose, establishing high osmotic pressures, can reduce the transport of water and nutrients from the base to the aerial part. Thus, the reduction of mineral elements in the MS medium, coupled with an increase in the amount of sugar in the growing medium and thus the reduction of nutrients to the leaf organs, would result in mineral stress. The leaf shoots, to cope with this state of stress, will emit roots. Authors [25] [26] have also reported the induction of roots by reducing the concentration of nutrients in the medium.

5. Conclusion

The potential use of grafted plants as sources of "reinvigorated" bud material for rapid clonal micropropagation is an advantage. Cotyledonary nodes in the presence of 2.2 mg/L BAP resulted in better proliferation rates. Also, coconut water (150 mL) promotes shoot elongation. Reducing the salt concentration, increasing the sucrose concentration, and using a liquid medium induced the best rooting rates.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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