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In vitro culture of cashew (*Anacardium occidentale* L.): influence of disinfection, antioxidants and carbon source on germination and growth

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ABSTRACT

This study aimed to determine the impact of disinfection protocol, antioxidants, and carbon sources on the germination and growth of cashew plants grown *in vitro*. Two disinfection techniques, single and double disinfection, were used on cashew seeds. The seeds were then grown on a medium supplemented with antioxidants and carbon source at different concentrations. The results showed that double disinfection resulted in the lowest contamination rates of 5.29% and 9% for calcium hypochlorite and sodium hypochlorite, respectively. Germination rates, mean germination times, and viable seedling rates were not affected by the disinfection technique nor type of disinfectant used. However, plants from seeds that underwent double disinfection had the highest growth rates. The presence of antioxidants and the interaction between antioxidants and their concentrations did not have a significant influence on germination rates, normal seedling rates, or mean germination time. On the other hand, these parameters were affected by the carbon source used. Overall, this study provides valuable insights into the factors that affect cashew seed germination and growth in.

Keywords: *In vitro*, disinfection, cashew, germination, growth.

INTRODUCTION

Native to Latin America, the cashew tree is cultivated to trade its nuts. The cashew kernel is used as a raw material in the food (chocolate, confectionery), pharmaceutical and cosmetics industries (Aliyu, 2007; Dosso et al., 2021). This production was estimated at over 3000000 t in 2016, with Côte d'Ivoire, India, Vietnam and Brazil being the main producing countries. Ivorian production was estimated at 700000 t in 2015 (Adaman and N'dri, 2016) and 968 676 tonnes in 2021 (CCI France Côte d'Ivoire, 2022).

This increase in national production is due to the growth in cultivated areas, which rose from 265654 ha in 2000, to 1350000 ha in 2018 (FIRCA, 2018; Timité et al., 2023). This galloping increase in cultivated areas can lead to a shortage or insufficiency of arable land and land disputes. Nut yields in Ivorian orchards remain low, at around 350 to 500 kg/ha, due to plantations created with unimproved planting

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material (Aliyu and Awopetu, 2007). Plantations are mainly direct seeded or planted cashew nut (Massai et al, 2021). Seedlings germinating from the same cashew tree are potentially different from one another.

Several vegetative propagation techniques (layering, cuttings, grafting) have been used in cashew trees (Kouakou et al., 2021; Djaha et al., 2022). However, these techniques do not allow mass production of planting material (Aliyu, 2005).

In addition to these techniques, *in vitro* culture of plant tissues and organs can also be used. These methods do not require the use of large cultivable areas, and enable improved varieties to be obtained in a short space of time, resistant to various pest attacks and with organoleptic characteristics to suit consumer tastes. Biotechnology is a powerful tool for overcoming natural barriers, and thus represents an alternative and complementary approach to conventional breeding efforts (Kouakou et al., 2021). But cashew trees, like most plants in the Anacardiaceae family, are highly resistant to *in vitro* propagation techniques. One of the major constraints *to in vitro* culture of forest species is contamination. Organs harvested in the field are difficult to disinfect. The success rate of disinfected explants is very low (3 to 25%). Most explants that survive after disinfection turn brown or necrotize after 20 days in culture (Nanti, 2020). To overcome the difficulties associated with disinfecting explants taken from mature trees in the field, vitroplants are increasingly being used as a source of explants. Cashew tree germination has been reported by Nanti (2020). However, to our knowledge, the influence of disinfection protocol on the one hand, and the effect of antioxidants and carbon sources on the other, on cashew seeds germination success and growth, have not yet been evaluated. The aim of the present work was to determine the effect of the disinfection protocol, antioxidants and carbon sources on *in vitro* seed germination and the growth of cashew seedlings.

MATERIALS AND METHODS

Study site

Manipulations were carried out in Abidjan, at University Nangui Abrogoua (former University Abobo-Adjame) research station (05°23´N, 04°00´W, forest zone). These nuts were harvested from a tree considered a high producer, in a village plantation plantation in Gohitafla, a town in west-central Côte d'Ivoire.

Simple disinfection

After 72 h soaking in tap water, the seeds were stripped of their hulls. Under a laminar flow hood, the naked seeds (without hulls) were soaked in 70% alcohol for 1 min, then immersed for 10 min in a 3.5% calcium hypochlorite solution (containing three drops of tween 20) or in a sodium hypochlorite solution with 1.2% active chlorine (containing three drops of tween 20). The seeds were then rinsed five times with sterile distilled water to remove the disinfectant. Using tweezers and scalpels, the disinfected seeds were stripped of their seed coat (Figure 1).

Double disinfection

Under a laminar flow hood, seeds were soaked in 70% alcohol for one minute, then immersed for 30 min in 7% calcium hypochlorite (with three drops of tween 20) or in sodium hypochlorite with 2.4% active chlorine (containing three drops of tween 20). Seeds were then rinsed five times with sterile distilled water, and left in sterile distilled water for 72 h. At the end of the soaking time, the seeds were removed from the water and disinfected a second time under the same conditions as before, this time with an immersion time of 15 min in 7% calcium hypochlorite or in sodium hypochlorite with 2.4% active chlorine. The seeds were then rinsed five times with sterile distilled water and

stripped of their shells and integument to release the kernels (Figure 2).

Influence of antioxidants on *in vitro* seed germination and cashew *in vitro* plant growth

Three antioxidants, activated charcoal (AC), ascorbic acid (AA) and polyvinylpyrrolidone (PVP), were used at different concentrations in this study (0; 1; 2 and 3 g/l). These substances were combined with the seed germination medium. The experiment was repeated three times for each antioxidant, using ten seeds per antioxidant concentration

Influence of carbon sources on *in vitro* seed germination and cashew *in vitro* plant growth

To optimize energy supply to the seed, three carbon sources were tested separately. These were: glucose, sucrose and fructose. These sugars were added to the MS culture medium at concentrations of 30, 60 and 90 g/l.

Seed cultivation

After surface disinfection, the seeds were halved and the embryo part (Nanti et al., 2018) was sown on MS medium (Murashige and Skoog, 1962), at a rate of 10 seeds per disinfection technique (Figure 3). Incubation took place in the culture room. After 16 days of culture, seed germination was assessed. The experiment was repeated three times.

***In vitro* culture conditions**

All experiments were carried out in a laminar flow hood, pre-cleaned with 70% alcohol. All equipment was autoclaved. Cultures were incubated in a culture room at a temperature of 25°C, a photoperiod of 12 h, a hygrometry of 70% and a light level of 100 $\mu E.m^{-2}.sec^{-1}$.

Statistical analysis

The experimental values obtained were subjected to an analysis of variance with one or two or two-criteria analysis of variance, using STATISTICA 7.1 software. A transformation $\arcsin(\sqrt{p})$ transformation (p = proportion) was performed before the analysis of variance of germination rates. and viable plants in order to normalize the values. This analysis was completed by a comparison of means using the Student Newman-Keuls test at 5% threshold.

Figure 1: Different stages in the simple surface disinfection of cashew seeds.
(a) cashew seeds soaked in water to soften hulls; (b) hulled seeds obtained after 72 h soaking in water; (c) hulled seeds; (d) hulled seeds soaked in disinfectant; (e) hulled kernels after disinfection.

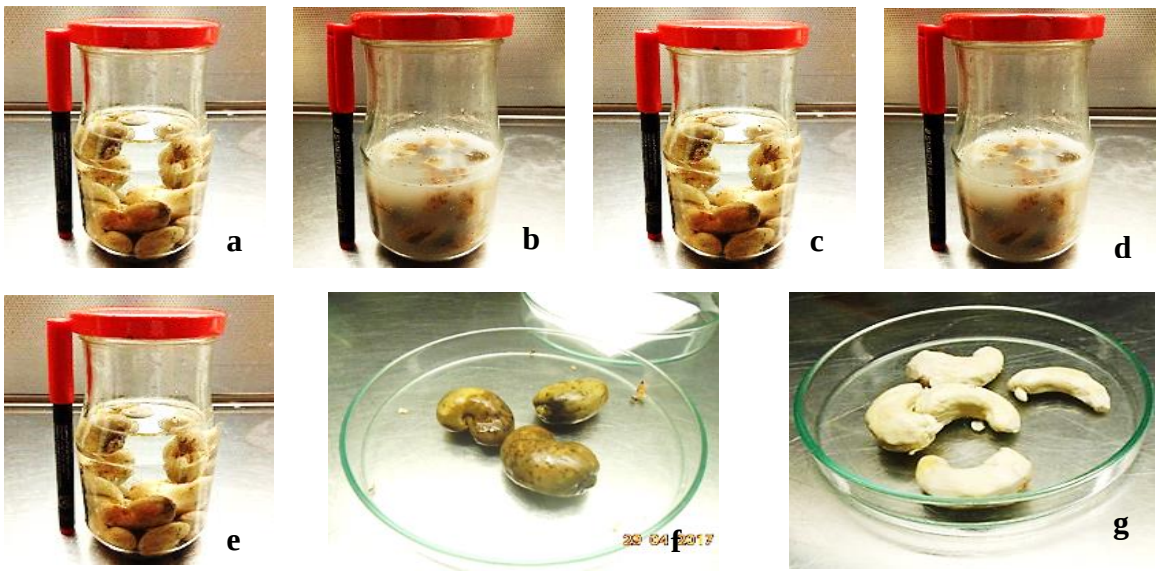

Figure 2: stages of double disinfection.
(a) seeds soaked in 70% alcohol (1 min); (b) seeds immersed in 7% calcium hypochlorite (30 min); (c) seeds thoroughly rinsed in sterile distilled water; (d) seeds immersed in 7% calcium hypochlorite (15 min); (e) seeds Influence of antioxidants on in vitro seed germination and cashew in vitro plant growth.

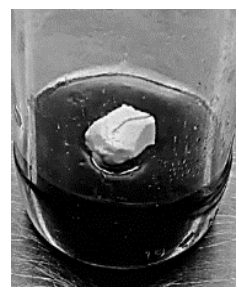

Figure 3: Half almond on MS medium containing activated charcoal.

RESULTS

After 16 days in culture, the seeds germinated to produce vitroplants (Figure 4).

Effect of disinfection technique

Experimental results relating to the disinfection technique are shown in Table 1. Analysis of the results showed a significant effect of the disinfection technique on the contamination rate, according to the Student Newman-Keuls test at the 5% threshold. The lowest contamination rates (5.29 and 9%, respectively for calcium hypochlorite and sodium hypochlorite) were obtained with seeds subjected to double disinfection. Seeds disinfected once with calcium and calcium hypochlorite recorded contamination rates of 22% and 30% respectively.

The results also showed that there was no difference between germination rates, mean germination times and viable plant rates, irrespective of the disinfection technique and type of disinfectant used ($P > 0.05$). Germination rates ranged from 76.6% to 90.2%. Average germination times ranged from 3.44 to 4.02 days. Viable seedling rates ranged from 46 to 52%.

Effect of antioxidants on *in vitro* seed germination and growth of cashew *in vitro* plantlets

Germination time, germination rates and normal seedling rates were not significantly influenced by the nature of the antioxidant ($P > 0.05$). The highest germination and normal plant rates were obtained with concentrations of 1 and 2 g/l for all antioxidants. Above these concentrations, and for media devoid of antioxidants, a decrease in germination and normal seedling rates was observed (Table 2). The results for antioxidant-dependent plant growth are shown in Table 3. A significant effect of antioxidants and their concentrations was observed ($P < 0.05$) on average plant size. The best growth rates were obtained with concentrations of 1 and 2 g/l activated carbon. On these media, seedlings grew to over 6 cm. Above these concentrations, growth in terms of height was poor.

Effect of carbon sources on *in vitro* seedling and growth of cashew

Concerning the influence of carbon sources, the different types of sugars incorporated to the culture medium, showed a variability of response on *in vitro* seedling in cashew (Table 4). The 30 g/l sucrose concentration was more favorable to germination (Table 4) and *in vitro* plant growth (Table 5). In addition, this concentration ensured good plant morphology. On the other hand, high doses (90 g/l) clearly slowed down the growth of vitroplants.

Figure 4: Cashew *in vitro* plant development stages.
(a) germinated seed after one week's culture; (b) 16-day-old cashew *in vitro* plant.

Table 1: Effect of disinfection technique on cashew *in vitro* plant growth.

Disinfecting agents	Type of disinfection	Mean germination time (d)	Contamination rate (%)	Germination rate (%)	Viable seedling rate
Calcium hypochlorite	Single disinfection	3.49 ± 0.31	30 ± 6.39 [a]	90.2 ± 19.38	46 ± 6.78
	Double disinfection	33.77 ± 0.29	9 ± 5.01 [b]	81 ± 5.01	51 ± 8.28
Sodium hypochlorite	Single disinfection	4.02 ± 0.25	22 ± 5.83 [a]	80 ± 4.26	47.65 ± 7.82
	Double disinfection	3.44 ± 0.25	5.29 ± 3.69[b]	76.76 ± 5.55	52 ± 6.70
probability	P1	0.71	0.31	0.55	0.85
	P2	0.59	0.001	0.61	0.96
	P3	0.13	0.70	0.80	0.52

In the same column, means followed by the same letter are significantly identical at the 5% threshold (Student Newman-Keuls test). Mean ± standard error; (P1): probability associated with type of disinfectant; (P2): probability associated with type of disinfection; (P3): probability associated with interaction between type and disinfectant.

Table 2: Effect of antioxidants and antioxidants concentrations on *in vitro* germination of cashew seeds.

Antioxidants	Concentrations (g/l)	Germination rate (%)	Normal plant rate (%)	Germination time (days)
CA	0	63 ±7.66 [a]	42 ± 8.34 [a]	4.6 ± 7.66 [a]
	1	72 ±6.68 [a]	63 ± 7.66 [a]	4.3 ± 6.68 [ab]
	2	81 ± 5.01 [a]	63 ± 7.66 [a]	3.76 ± 5.01 [b]
	3	57 ± 8.05 [a]	42 ± 8.33 [a]	4.67 ± 8.05 [a]
AA	1	63 ± 7.66 [a]	42 ± 8.34 [a]	4.36 ± 7.66 [ab]
	2	66 ± 7.39 [a]	51 ± 8.28 [a]	4.2 ± 7.39 [ab]
	3	51 ± 8.28 [a]	33 ± 8.05 [a]	4.87 ± 8.28 [a]
PVP	1	66 ± 7.39 [a]	48 ± 8.34 [a]	4.37 ± 7.39 [ab]
	2	69 ±7.7 [a]	51 ± 8.28 [a]	4.33 ± 7.07 [ab]
	3	57 ± 8.05 [a]	39 ± 8.28 [a]	4.77 ± 8.05 [a]
Probability	P1	0.37	0.140	0.204
	P2	0.03	0.027	< 0.001
	P3	0.92	0.935	0.647

In the same column, means followed by the same letter are significantly identical at the 5% threshold (Student Newman-Keuls test). Mean ± standard error; P1: probability of antioxidant type; (P2): probability of antioxidant concentration; (P3): probability of antioxidant type-concentration interaction;CA: Activated Carbon; AA: Ascorbic Acid; PVP: Polyvinylpyrrolidone.

Table 3: Effect of antioxidants and antioxidants concentrations on cashew *in vitro* plant growth.

Antioxidants	Concentrations (g/l)	Average seedling size (cm)	Number of leaves
CA	0	4.82 ± 0.34 [b]	4.2 ± 0.13 [b]
	1	6.93 ± 0.30 [a]	5 ± 0.18 [a]
	2	7.78 ± 0.27 [a]	5.03 ± 0.11 [a]
	3	4.49 ± 0.32 [b]	3.97 ± 0.14 [b]
AA	1	5.42 ± 0.32 [b]	4.27 ± 0.13 [b]
	2	5.23 ± 0.35 [b]	4.37 ± 0.13 [b]
	3	4.36 ± 0.30 [b]	4.1 ± 0.13 [b]
PVP	1	5.64 ± 0.30 [b]	4.4 ± 0.12 [b]
	2	5.40 ± 0.35 [b]	4.4 ± 0.11 [b]
	3	4.53 ± 0.30 [b]	4.27 ± 0.12 b
Probability	P1	< 0.001	< 0.001
	P2	< 0.001	< 0,001
	P3	0.11	< 0,001

In the same column, means followed by the same letter are significantly identical at the 5% level (Student Newman-Keuls test). Mean ± standard error. (P1) : probability of antioxidant type ; (P2) : probability of antioxidant concentration ; (P3) : probability of antioxidant type-concentration; CA: Activated Carbon; AA: Ascorbic Acid; PVP: Polyvinylpyrrolidone.

Table 4: Effect of carbon sources and carbon sources concentrations on *in vitro* germination of cashew seeds.

Carbon source	Concentrations (g/l)	Germination rate (%)	Normal plant rate (%)	Germination time (days)
Sucrose	30	81 ± 5.01 [a]	51 ± 8.28 [a]	4.2 ± 0.17 [b]
	60	69 ± 7.07 [a]	45 ± 8.36 [a]	4.67 ± 0.17 [ab]
	90	57 ± 8.05 [a]	42 ± 8.34 [a]	4.8 ± 0.17 [ab]
Glucose	30	75 ± 6.22 [a]	48 ± 8.4 [a]	4.23 ± 0.16 [b]
	60	60 ± 7.88 [a]	45 ± 8.36 [a]	4.7 ± 0.17 [ab]
	90	54 ± 8.19 [a]	42 ± 8.34 [a]	4.8 ± 0.17 [ab]
Fructose	30	69 ± 7.07 [a]	45 ± 8.36 [a]	4.37 ± 0.15 [ab]
	60	63 ± 7.66 [a]	42 ± 8.34 [a]	4,67 ± 0,17 [ab]
	90	60 ± 7.88 [a]	39 ± 8.29 [a]	5 ± 0.20 [a]
Probability	P1	0.558	0.829	0.651
	P2	< 0.010	0.587	< 0.001
	P3	0.858	0.999	0.963

In the same column, means followed by the same letter are significantly identical at the 5% threshold (Student Newman-Keuls test). Mean ± standard error; P1: probability of Carbon source ; (P2): probability of concentration; (P3): probability of Carbon source - concentration interaction

Table 5: Effect of carbon source and carbon sources concentrations on *in vitro* germination of cashew seeds.

Carbon source	Concentrations (g/l)	Number of leaves	Average seedling size (cm)
Sucrose	30	5 ± 0.12 [a]	5.75 ± 0.39 [a]
	60	4.37 ± 0.13 [bc]	4.95 ± 0.28 [ab]
	90	4.07 ± 0.16 [bc]	4.39 ± 0.22 [cd]
Glucose	30	4.47 ± 0.12 [b]	5.22 ± 0.33 [ab]
	60	4.3 ± 0.13 [bc]	4.55 ± 0.31 [bc]
	90	4 ± 0.14 [bc]	3,67 ± 0.21[cd]
Fructose	30	4.4 ± 0.10 [bc]	4.94 ± 0.32 [ab]
	60	4.33 ± 0.13 [bc]	4.38 ± 0.24 [cd]
	90	3.87 ± 0.15 [c]	3.41 ± 0.18 [d]
Probability	P1	0.027	0.002
	P2	< 0.001	< 0.001
	P3	0.192	0.967

In the same column, means followed by the same letter are significantly identical at the 5% level (Student Newman-Keuls test). Mean ± standard error. (P1) : probability of the carbon source; (P2) : probability of the concentration ; (P3) : probability of the carbon source-concentration interaction.

DISCUSSION

Sodium hypochlorite and calcium hypochlorite have often been used for in vitro disinfection of explants. The efficacy of these disinfectants has been variously reported (Nanti, 2020; Koné et al., 2022). In this study, the disinfectant had no effect on contamination reduction. The lowest contamination rate was obtained with seeds that had undergone double disinfection. This is explained by the fact that the first disinfection would have eliminated only surface pathogens. After soaking, the hulls became soft and permeable to water. Thus, the second disinfection would be in-depth. In other words, disinfection of the kernel to eliminate endogenous pathogens. Average germination times ranged from 3.44 to 4.02 days. Germination in this study was earlier than that obtained by Djaha et al. (2022). These authors obtained an average *in situ* germination time of between 5 and 8 days. Double disinfection was also used to disinfect node segment explants by Mokea-Niaty et al. (2017). This author observed a reduction in the rate of bacterial and fungal contamination of *Alchornea cordifolia* by the application of double disinfection. In fact, germination depends on the physiological state of the seed and the hydration of the embryo. Germination begins with the imbibition of seed tissue, characterized by the absorption of water from the external environment (Gimeno-Gilles, 2009). Meyer et al. (2004) have shown that water causes cells to swell and divide.

The absence or very low incorporation of antioxidants in the culture medium did not inhibit the accumulation of phenolic compounds. Indeed, the oxidation of phenolic compounds produces toxic substances that would be detrimental to the survival of explants under *in vitro* culture conditions (Ozyigit et al., 2007). Roussos and Pontikis (2001) and Arnaldos et al. (2001) have shown that the accumulation of phenolic compounds leads to browning and eventual death of explants. Seeds contain phenolic compounds and in culture they excrete these compounds into the medium, where they oxidize. These oxidized compounds can bind to nutrients and make them unavailable to plants, resulting in poor growth.

These results are similar to those obtained by Scofield et al. (2007) for rice L. These researchers reported that sucrose affects growth equilibrium and mitosis. They pointed out that high concentrations of sucrose, inducing high osmotic pressures can reduce the transport of water and nutrients from the root to the aerial part. Sucrose has always been considered to be the best source of carbon in cell and tissue culture media, since it is the main sugar that transits the phloem of many plants (Scofield et al., 2007).

Conclusion

The overall aim of this study was to determine the effect of the disinfection protocol and antioxidants on *in vitro* seed germination and growth of cashew seedlings. Double disinfection of seeds with calcium or sodium hypochlorite greatly reduced contamination rates. Activated charcoal (0.2%) and sucrose (3%) are respectively the most suitable antioxidant and carbon source for obtaining plantlets in quantity and quality.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

The protocol for this study was established by BTJ-IN. Field harvesting of cashew nuts was carried out by KMK. Statistical analyses of the data were carried out by GYG and SHK. BTJ-IN and SHK participated actively in the drafting of this manuscript. All work was supervised by MK.

All authors have given their agreement to the final version of the manuscript and its publication.

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