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PATHOLOGICAL CHALLENGES AND EFFECTS OF DISINFECTION ON THE GROWTH OF CEDAR (*Cedrela odorata* L.*)* SEEDSIN *IN-VITRO* PROPAGATION

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ABSTRACT

Cedrela odorata is mostly propagated through seed, but the process is constrained by frequent infections due to seed damping. To generate a disease free planting material to enhance reforestation process and mitigate the effect of global climate change, *in-vitro* propagation methods is imperative. Plantlets of *C. odorata* were raised from seeds disinfected using the single and double disinfection methods and contaminants were identified using Barnett and Hunters manual. The uninfected plantlets were sub-cultured in MS medium, supplemented with plant growth regulator (PGR) regimes(BAP + IBA) –A(1.5mg/L + 0.0mg/L); B(2 mg/L +0.5mg/L); C(2.5mg/L+1.0mg/L)and D(a PGR-free) MS medium in a completely randomized design with seven replicates. The number of nodes, leaves, roots and contamination percentages were collected and analysed using Analysis of variance and means separatedusing least significant difference (LSD) at 5 % significance level. A high rate of fungal contamination was observed within seven days of propagation. The contaminants were identified as *Fusarium verticilloides, Aspergillus niger, Colllectotricum spp.* 1.5 mg/L of BAP gave the best shoot development. The apical and nodal cuts germinated faster with rapid leaf production and root formation after 13 days of inoculation into media not supplemented with PGR after disinfection.Root was produced in D(0.43) two weeks after inoculation, shoot length range from B(2.19cm) to A(3.30cm) while the number of nodes and leaves rangedfrom B(1.36) to A(4.43) and B(2.43) to A(13.43) respectively

Keywords: Disinfection, contamination, regeneration, exposure time, growth regulators.

Introduction

In vitro techniques of raising aseptic or clean plantlets , aside from the mother plant or seeds in an artificially prepared nutrient medium is important for mass propagation especially when sometimes seed propagation and conventional vegetative methods are difficult or not successful (Babaei *et al.,* 2013). Aseptic condition are maintained to obtain disease free or clean plantlets and often time's contamination is a major setback in achieving this process (Omamor *et al.,* 2007), as this is not visible when cultures are established at the initial stage as most contaminants could be internal or acquired from the surrounding where the cultures are prepared (Reed *et al*., 1998; Babaei *et al*. 2013).

Several types of *in-vitro* culture contaminants include a variety of fungi, bacteria, and viruses. Fungi have been the most common and easily recognized at glance in *in-vitro* culture vials as microbes that cause many *in-vitro* plant culture contaminations (Mng'omba *et al*., 2012). These fungi contaminants could be endogenous, cryptic or endophytic to the plants (Herman, 1990; Mng'omba *et al*., 2012). Endogenous or endophytic fungi are difficult to decontaminate, meanwhile in the wild this microbes are beneficial for the growth and development of the plants (Mng'omba *et al*., 2012).

Seed borne pathogens, have been responsible for the rapid loss of viable seeds as these pathogens do not enhance the proper germination of seeds (Orwa *et al*., 2009). This will eventually lead to

deforestation as explants for propagation soon become inadequate to sustain plantation development. Eradication of pathogen from plants is cumbersome, pathogens are associated with them such that a selective treatment of eliminating them also directly or indirectly affects the plants (Abshukor and Ejainol, 1997).

Surface sterilizers have been described as chemicals that render plants free from pathogens and act on the surface of the plants thus increasing the wetting ability, such surface sterilizers are Tween 20, 70% ethyl alcohol, teepol, 5% and/ or 10% sodium hypochlorite among others. (Mng'omba *et al*., 2012).These when used rids the explants of the pathogens and can ensure that clean plantlets are raised.

Cedrela odorata is a large tropical treewhich belongs to the family Meliaceae and valued for its quality, ductility and durability (Salazar *et al*., 2000; Orwa *et al*., 2009).

IUCN (2014) reported this species to be threatened and going extinct. Interest in *C. odorata* as source of plywood production for furniture, medicinal value, and bee keeping, qualifies it as economically valuable (Millán-Orozco *et al*., 2011;Díaz-Quichimbo *et al*., 2013).

Tissue culture techniques or *in-vitro* propagation is good for production of large clonal plants, pathogen free stock, germ-plasm conservation and mass propagation independent of seasons (Jaenicke and Beniest, 2001)

When seeds are viable, germination of this species is rapid and this arouses the interest for its mass propagation through *in-vitro* technique rather than the conventional method of propagation.

Wilting, die backs have also been reported in young plants that have grown due to attack by fungal pathogens which mitigates the reforestation of trees (Millán-Orozco *et al*., 2011).

Mass propagation of this viable economic tree *C. odorata has* become necessary for plantation establishment for its immense benefit to man and environment (Valverde-Cerdas *et al*., 2008) Therefore, one of the constraint of tissue culture is the high rate of contamination encountered, in an attempt to raise clean and disease free plantlets for mass propagation and distribution to the end users is thus the aim of this study.

.Materials and Methods

Collection of Explants

Seeds, young shoot tips and nodal cuttings of *C. odorata* were collected from the nursery and arboretum of Forestry Research Institute of Nigeria between the months of February and March for this study. The seeds were stored in the refrigerator until needed while the cuttings were cut first thing in the morning when needed and washed for 30minutes under flowing tap water.

Media Preparation

Explant disinfection medium

Standard methods for media preparation was used to prepare Murashige and Skoog (MS) media (Murashige and skoog, 1962). Nutrient Agar (NA) and Potatoes Dextrose agar (PDA) were also prepared fresh when needed from the powdered form according to manufacturer's specification. NA and PDA were used to culture contaminants observed in the course of *in-vitro* propagation of *C. odorata*. All media were sterilized at 115^{o}C for 15 minutes.Microbial growth were cultured on plates of potatoes dextrose agar (PDA) supplemented with Streptomycin (1mgL^{-1}) and incubated at room temperature for four to seven days (4-7days). They were subcultured to get pure colonies of the contaminants for identification.

Disinfection of Explants

Seeds of *C. odorata* were indexed to test for the wholeness in terms of pathogens using the indexing media (sucrose 10 gl^{-1}, casein hydrolysate 8 gl^{-1}, Yeast 4, KH_2PO_4 2 $gl^{-1.}$, $MgSO_4.7H_2O$ 0.15gl^{-1}, agar 10 gl^{-1}). Seeds of *C. odorata* were surface sterilized using the single and double method, followed by immersion in 10%w/v of the fungicide carbendazim at varying exposure time 1, 3, 5 and18 hours, rinsed in four (4) changes of sterile distilled water to remove the fungicide before inoculation into media. Also nodal cuttings and shoot tips from the wild were also collected first in the morning and cleaned under a flowing tap water for 30minutes to remove any dirt, transferred into the lamina flow hood, the single and double method of disinfection was used, followed by exposure to 1% carbendazim for 5, 15, 25 and 40minutes, rinsed in four changes of sterile distilled water before inoculation. Plants were transferred into the growth room at 16/8 hour photoperiod.

Growth culture and subculture medium compositions

Subculture of *in-vitro* raised plantlets, was carried out using the MS media at full strength supplemented with BAP(Benzyl Amino Purine) and IBA(IndolebutyricAcid) for its propagation**.**MS medium was prepared at full strength and supplemented with (BAP + IBA) -A (1.5 mg/L + 0.0 mg/L); B (2 mg/L +0.5 mg/L), C (2.5 mg/L+1.0mg/L), D (0.0 mg/L +0.0 mg/L)

Experimental design

The two experiment were factorials with two factors at four levels; the first was two disinfection methods, single and doubleat four different times, while the second was two explant type at four levels of plant growth regulators.

The cultures were arranged in a completely randomized design(CRD) with seven replicates.

Identification of Contaminants

Identification of the fungal contaminants was by inferring based on cultural and morphological characteristics using Barnett and Huntersmanual of fungal identification.

Data collection and Analysis

Data collected include, shoot length, number of roots, number of leaves and nodeswere subjected to Analysis of variance (ANOVA) using SAS 9.0 version, means were separated using least significant difference (LSD). Qualitative and quantitative assessment of the contaminated tubes was also carried out.

Results

Nodal cuttings from the wild exposed to 1% carbendazim, at varying time for 5, 15, 25 and 40minutes showed no morphogenic response as complete blackening of the cuts with no growth was observed one week after inoculation.

Evaluation of the effect of the disinfection time on the rate of survival of germinated seeds (Table1) indicated a higher rate at 1 and 3 hours for both single and double disinfection with no significant difference in their means

Table 1: Effect of Disinfection Time and Method on the Root and Shoot Development of *Cedrela odorata* seeds

Factors	Levels	Root				Shoot			
		1 WAI	2 WAI	3 WAI	4 WAI	1 WAI	2 WAI	3 WAI	4 WAI
Disinfection time(DT)	1	0.71a	1.47a	2.97a	0.72ab	0.71a	0.82a	2.73a	2.39a

	3	0.71a	1.37a	3.46a	0.57b	0.71a	0.87a	3.02a	3.05a
	5	0.41c	0.82a	1.46a	0.60ab	0.57b	0.65a	1.38a	2.71a
	18(ON)	0.57b	0.74a	I.85a	1.01a	0.57b	0.61a	1.81a	2.35a
Disinfection method (DM)	Single (S)	0.64a	0.97a	2.21a	0.68a	0.64a	0.70a	2.11a	2.34a
	Double(D)	0.56b	1.07a	2.67a	0.77a	0.64a	0.77a	2.36a	2.86a
LSD (p = 0.05)									
DT		0.08*	0.64	2.39	0.42	0.00*	0.36	2.16	2.53
DM		0.06*	0.45	1.69	0.31	0.00	0.25	1.52	1.79
DM X DT		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

ON- overnight,Means with the same alphabets down the column are not significantly different from each other at 5% level of probability

Subculture of the grown disinfected seeds into MS medium supplemented with different concentration of the same plant growth regulators are shown in tables 2 and 3 over a period of 4weeks.Root production was observed only in the control (D), while shoot production was highest in A with BAP only at 1.5mg/L.

Table 2: Effects of BAP/IBA Combinations and Explant Types on Number of Roots and Shoot Length of *Cedrela odorata* at Successive Growth Weeks

Factors	Levels	Number Of Roots				Shoot Length(cm)			
		1 WAI	2 WAI	3 WAI	4 WAI	1 WAI	2 WAI	3 WAI	4 WAI
Growth regulators (GR)	A	0.00b	0.00	0.00b	0.00b	3.01a	3.02a	3.27a	3.30a
	B	0.00b	0.00b	0.00b	0.00b	2.19b	2.25b	2.27b	2.19b
	C	0.00b	0.00b	0.00b	0.00b	2.29b	2.36b	2.43b	2.48b
	D	0.29a	0.43a	0.36a	0.43a	1.89b	2.07b	2.29b	2.38b
Explant types (ET)	Shoot tips	0.11	0.18	0.14	0.21	2.56	2.74	2.86	2.95
	stem	0.03	0.04	0.04	0.00	2.12	2.11	2.27	2.23
LSD (p = 0.05)									
GR		0.18*	0.17*	0.18*	0.29*	0.53*	0.62*	0.82	081*
ET		0.12	0.12*	0.12	0.20*	0.38*	0.44*	0.58*	0.57*
GR X ET		0.00	0.00*	0.00*	0.00*	0.00	0.00	0.00	0.00

Means with the same alphabets down the column are not significantly different from each other at 5% level of probability. BAP+IBA combinations; A(1.5 mg/L + 0.0 mg/L); B(2 mg/L +0.5 mg/L), C(2.5 mg/L+1.0mg/L), D (0.0 mg/L +0.0 mg/L)

Table 3: Effects of BAP/IBA Combinations and Explant Types on Number of Nodes and Leaves of *Cedrela odorata* at Successive Growth Weeks.

Factors	Levels	Number of nodes				Number of leaves			
		1 WAI	2 WAI	3 WAI	4 WAI	1 WAI	2 WAI	3 WAI	4 WAI
Growth regulators (GR)	A	2.79a	3.93a	4.64a	4.43a	7.14a	11.86a	11.64a	13.43a

	B	0.93b	1.07b	1.71b	1.36b	2.43bc	3.21b	3.00b	2.43b
	C	1.43b	1.21b	1.57b	1.71b	0.86c	3.50b	3.50b	4.29b
	D	1.36b	2.36b	2.29b	2.50ab	4.71ab	6.21b	6.50b	7.00b
Explant types (ET)	Shoot tips	2.86	3.61	3.96	4.07	7.21	10.43	10.18	10.86
	stem	0.39	0.68	1.14	0.93	0.36	1.96	2.14	2.71
LSD (p = 0.05)									
GR		1.27*	1.57*	1.83*	1.99*	2.65*	5.16*	5.22*	6.05*
ET		0.90*	1.12*	1.29*	1.40*	1.87*	3.65*	3.69*	4.28*
GR X ET		0.00	0.00	0.00	0.00	0.00*	0.00	0.00	0.00

Means with the same alphabets down the column are not significantly different from each other at 5% level of probability. . BAP+IBA combinations; A (1.5 mg/L + 0.0 mg/L); B(2 mg/L +0.5 mg/L), C(2.5 mg/L+1.0mg/L), D (0.0 mg/L +0.0 mg/L)

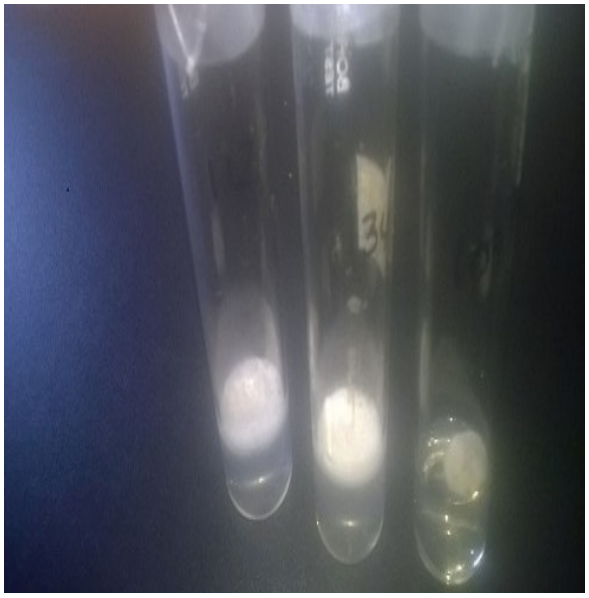

Plate 1a: Tubes of contaminated seeds a week after inoculation

Plate1b: Tube of contaminated seedling

Three genera of fungi contaminants were isolated from the seeds of *C. odorata* as shown in the plates A, B and C below with their probable identityto species level as *Fusarium verticilloides Collectotrichum spp*. and *Aspergillus niger*. (Barnett and Hunters, 2001)

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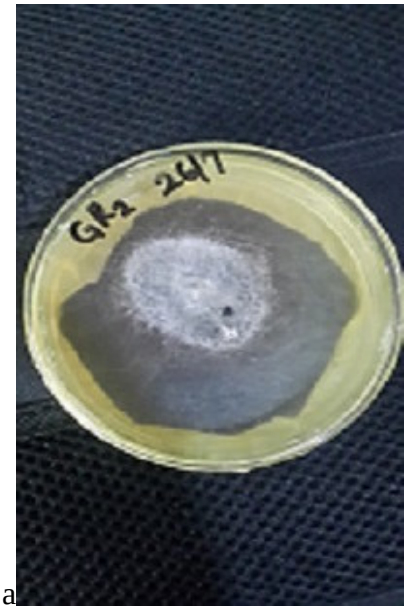
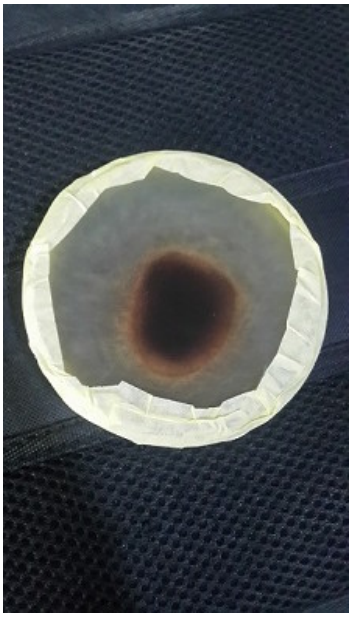
a  b 

Plate A: (a)Front View and (b) Back view of *Fusarium verticilloides*

Plate B; *Collectotrichumspp*

Plate C: *Aspergillusniger*

Discussion

Contamination of plantlets has been a constraint to tissue culture. Reports have it that seeds of *C. odorata*possess seed borne pathogens, which often manifest after days to weeks of regeneration, plantlets were observed to wilt and eventually die off (Millán-Orozco *et al*., 2011). Amixed colony of organisms were noticed in the tubes few days after inoculation of the seeds and in growing plantlets. Although surface disinfection was carried out using the single and double method to rid the seeds of pathogen. This was found to be inadequate, so further disinfection through chemical means was adopted using the fungicide Carbendazim. This was observed as shown

in Plates 1a, b, A, B and C. It corroborates the reports of Millán-Orozco *et al*., (2011).

Carbendazim is a broad spectrum benzimidazolecarbamate fungicide possessing both a surface and systemic activity (ACP, 1992).It was applied at 10% w/v and the seeds were immersed at varying time, although for those that had prolonged contact time 5hrs and 18hrs the tube appeared clean with fewer fungal or bacterial growth while most tubes were without contaminants. However radicle emergence was noticed after 5days but did not grow beyond that, this effect observed could be likened to the findings of Truta *et al*. (2010) who reported the effect of carbendazim on the seeds of fenugreek pretreated with 0.1, 0.2, 0.5 and 1.0% and reported that the decrease in height and inability to grow tall could have been attributed to the presence of cytotoxic compound causing the inhibition of DNA synthesis and also cell death.

In-vitro propagation of *C. odorata* had been achieved mainly from seeds, propagation using nodal cuts from matured plants proved difficult with oxidation and death of the explants after disinfection and inoculation, this is in line with findings of Garcia-Gonzales *et al.*, (2011).

The survival of the seeds was very low owing microbial contamination that were observed. Disinfection using carbendazim increased germination rate, however, it was observed that rate of germination in terms of root and shoot development was higher in plantlets that had contact time at 1 and 3 hours.

Orwa *et al*, (2009), reported that pathogens such as *Fusarium sp.* implicated in wilting of this species. This was observed in this findings as one of the identified contaminant was *Fusarium verticilloides*

Pathogens that hindered the survival/viability of the seeds were identified as *Fusarium verticilloides*, *Collectotrichum* sp. This is in line with the finding of Orwa *et al*., (2009) as a mixed colony of organisms were found to inhabit the seeds of *Cedrela odorata*.

Subculture of plants in Murashige and Skoog media supplemented with PGR; BAP and IBA indicated that species of *C. odorata* can be regenerated successfully in PGR free media, this finding is in line with that of Garcia-Gonzales (2011). PGR is not required and even now they are expensive and the world is tending towards the use of organic materials (Mng'omba *et al*., 2012).

Findings reported by several research group on tissue culture of woody plants suggested that surface sterilization alone was not sufficient to produce clean cultures explants, but that when fungicides and/or antibiotics are added to culture media best results were obtained in controlling contaminants from bacteria and fungi (Eed *et al*., 2010). This was not the case in this experiment as the fungicides were not added to the culture media, however the explants were exposed to fungicides at the same concentration but with varying exposure time and clean culture were obtained.

Conclusion

Healthy interaction of plants and microbes should be sought after to prevent losing them to the harmful activities of some microorganisms that cause disease.

Successful regeneration of *C. odorata* with seeds was achieved with disinfection using Carbendazim. *In-vitro* techniques has helped to raise high quality plantlets which can be made available to farmers. However, recalcitrance to *in-vitro* culture due to fungi and bacteria, oxidative response of tissues after disinfection, low response of explants gives room for more research in *in-vitro* culture techniques.

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