Journal of Forestry Research and Management. Vol. 16(3).1-11; 2019, ISSN 0189-8418

www.jfrm.org.ng

DISINFECTION PROTOCOL FOR IN-VITRO CULTURE INITIATION OF *Vitellaria paradoxa* C.F. Gaertn.

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ABSTRACT

Vitellaria paradoxa is a tree cherished for its economic and medicinal value. However, the tree species has shown great difficulty in its propagation through tissue culture; as it is prone to contamination, slow growing and oxidation of its explant tissues. The use of recently developed Plant Preservative Mixture (PPM) and other potent anti-fungi such as Benlate are not within the reach. Therefore, an experiment was conducted to establish the effectiveness of the locally available fungicides and antibiotics on culture initiation of *V. paradoxa*. These were combined into 5 mixtures and 2 dipping time with 10 replications each and laid out in completely randomized design. Data collected include number of infected tubes, regenerative ability of the explants and lethal effect of the mixtures at 1 and 4 weeks after inoculation (WAI). Dipping in composition E (5 g/l Z-force + 5 g/l Cibaplus + 0.4 g/l Ciproxamed) overnight was more effective in reducing the level of infection as 10% and 30 % of the tubes got contaminated at 1 and 4 WAI. The same treatment had the highest number of regenerated explants; 100% and 60 % at 1 and 4 WAI, respectively. Moreover, all explants dipped in treatments A, C and E for 2 hours were not able to regenerate at 4 WAI. Dipping the explants in treatments C and E for 2 hours showed 0 % toxicity but with heavy contamination while dipping in treatment A for 2 hours showed the most toxic effects (70 %) at 4 WAI. It can be inferred from this study that dipping in treatment E overnight gave better results and hereby recommended for sterilization of wildly collected *V. paradoxa* shoot tips.

Keywords: Contamination, Culture initiation, Fungicides, Regenerative ability

Introduction

The importance of plant tissue culture in forestry mainly in the area of mass production of tree species cannot be over emphasised (Kumar, 2016). The seedlings of tree species of economic importance that are difficultly raised are easily propagated through the techniques irrespective of the season within a short time and limited space (Hussain *et al*., 2012).In Nigeria and especially Forestry Research Institute of Nigeria, the application of plant tissue culture techniques is at secondary level. The recent installation of Temporary Immersion Bioreactor system (TIBs) has just positioned the institute in a better way at solving any challenges relating to mass propagation of tree species of economic importance.

Notably, for a tree species to be in-vitro propagated, protocol has to be developed. This involves four stages via initiation, shoot induction, regeneration and multiplication, root induction and acclimatisation (Abdullahi, 2013).The success of any plant tissue culture practice stands on the availability of clean culture from initiation stage. Several techniques being employ to achieve this include culturing of seeds, explants collection from plant stock in the laboratory and screen-house, and lastly those collected directly from the wild. The use of seed is better among these choices but only practicable for some species such as *Nauclea didderichii, Eucalyptus calmadulences, Bombax Ceiba* and *Moringa oleifera* while external sourcing which is often faced with the

problem of heavy microbial contamination is applicable to others such as *Vitellaria paradoxa*, *Allamblankia florinbunda*, *Irvingia species* and many more (Ahmed *et al*., 2010; Yeboah *et al*., 2010).

Vitellaria paradoxa is a useful multipurpose and economic tree species of the African continent (Ugese *et al*.,2012). Its extracted oil known as Shea butter is an important raw material in food, cosmetic and pharmaceutical industries (Wara, 2011).The invaluable contribution of the tree to meeting diverse human needs made a case for its inclusion in ongoing national afforestation project in the country. However, the difficulty faced in propagating the species through seeds and vegetative means necessitated alternative method of its production by clonal method (Paul, 2012; Lovett and Haq, 2013).

Past attempts to raise *V. paradoxa* in-vitro in FRIN has been mainly hampered by microbial contaminations and slow growth. Recommended remedies such as the use of Benlate, Plant preservative mixture (PPM) and other effective anti-microbes that would have lessen the problems are but not within the reach (Simon *et al.*, 2012).This necessitated the trials of locally available fungicides and bactericides to establish a suitable disinfection protocol for successful culture initiation and mass production of the species.

Materials and Methods

Study site

The study was undertaken at the plant tissue culture laboratory of Biotechnology Section, Department of Bioscience, Forestry Research Institute of Nigeria (FRIN), Jericho Hill, Ibadan, Nigeria. The Institute is located on longitude 07°23'18″ to 07°23'43″N and latitude 03°51'20″ to 03°23'43″E (FRIN, 2018).

Treatments and experimental design

The experiment involved ten treatments made up of five different combinations of fungicides and bactericides (Table 1) at two times of Dipping (Overnight and 2 hours). The fungicide used; Z-Force (active ingredient; Mancozeb 80%) and Cibaplus (active ingredient; Imidacloprid 10% + Metalaxyl 10% + Carbendazim 10%) were added at the equivalent rate of their active ingredients. Each treatment were set up with 10 replications and laid out in completely randomised design.

Table 1: Components of antimicrobial formulations used

Reagents	Antimicrobial compositions (g/l)				
	A	B	C	D	E
Z-force	10		10		5
Cibaplus		10		10	5
Amoxycillin	0.4	0.4			
Streptomycin			0.2	0.2	
Penicillin			0.12	0.12	
Ciproxamed					0.4

A (10 g/l Z-force + 0.4g/l Amoxycillin); **B** (10 g/l Cibaplus + 0.4g/l Amoxycillin); **C** (10 g/l Z-force + 0.2g/l Streptomycin + 0.12 g/l Penicillin); **D** (10 g/l Cibaplus + 0.2 g/l Streptomycin + 0.12 g/l Penicillin); **E** (5 g/l Z-force + 5 g/l Cibaplus + 0.4 g/l Ciproxamed)

Medium preparation, Explants collection and Surface sterilization

Murashige and Skoog medium (50 % basal salts) was used for the experiment. 34.43g of MS powder with Vitamin and sucrose supplements (M5501, SXS5501015A) was measured per litre of distilled water (Table 2). The medium was basally added 2.0 mg/L GA_3 and 1.0 mg/l BAP, gelled with 4.0 g/l Agar (Sigma-Aldrich), homogenised, dispensed at 20 ml/tube of 50 ml, cover and sterilized at 121 °C and 15 *hpa* for 15 minutes.

Shoot tips of a well-managed *Vitellaria paradoxa* seedlings in the screen house were collected and used as explant. The explants were washed and flushed for 15 minutes under flowing tap. In order to prevent exudate, they were dipped in 0.3 % solution of ascorbic acid for one hour after which they were rinsed with sterile distilled water for 3 times. Thereafter, a portion of the explants were dipped into the prepared antimicrobial mixture stirred and left over night while the other portion were dipped for 2 hours on the day of inoculation (Table 1). Both portion were decanted and rinsed 4 times with sterile distilled water inside pre-surface sterilized laminar hood. They were then dipped in 70 % ethanol for 5 minutes, rinsed 3 times and dipped in 10 % hypochlorite with 2 drops of Tween 20 for 15 minutes. Thereafter, the explants were rinsed 4 times and blotted on petril dish laid dry sterilised filter paper. Each explant was trimmed and cut at a slant down to about 1.5 cm and inoculated on the medium. The tubes' cap were sealed with parafilm and placed in the growth room under 16/8 hours light and dark photoperiod.

Table 2. MS media composition

Components	MS Powder
Macronutrients (mg/l)	
Ammonium Nitrate	1,650
Calcium Chloride, Anhydrous	440
Magnesium Sulfate, Anhydrous	370
Potassium Nitrate	1,900
Potassium Phosphate Monobasic, Anhydrous	170
Micronutrients mg/l	
Boric Acid	6.2
Cobalt chloride, Hexahydrate	0.025
Cupric Sulfate, Pentahydrate	0.025
Manganese Sulfate, Tetrahydrate	22.3
Molybdic Acid Sodium Salt, Dihydrate	0.25
Potassium Iodide	0.83
Zinc Sulfate, Tetrahydrate	8.6
Iron source (mg/l)	
EDTA, Disodium, Dihydrate	37.3
Ferrous Sulfate, Heptahydrate	27.8
Vitamins (mg/l)	
Myo-inositol	100
Glycine	2
Nicotinic Acid	0.5
Pyridoxine, Hydrochloride	0.5
Thiamine Hydrochloride	0.1

Data collection and analysis

Effectiveness of the fungicide mixtures on microbial growth inhibition was determined in terms of number of infected tubes, Regenerative ability of the explants and lethal effects of the mixture on the explants. These were collated by counting and visual observation at first and fourth weeks after inoculation. Data were analysed descriptively using MS Excel 2013 version.

Results

Effect of Fungicides and antibiotics mixtures on microbial growth inhibition

The results of the use of different fungicide mixtures on microbial growth prevention in in-vitro culture of *V. paradoxa* is presented in Figures 1 - 4. It was discovered that treatment E(5 g/l Z-force + 5 g/l Cibaplus + 0.4 g/l Ciproxamed) provided better inhibition and had lowest level of infection as 10 % of the tubes were infectedwhen allowed to stay overnight. This was followed by 20 % in treatment A (10 g/l Z-force + 0.4g/l Amoxycillin) and 40 % in treatment C(10 g/l Z-force + 0.2g/l Streptomycin + 0.12 g/l Penicillin)at 1Week after inoculation (WAI) (Figure 1). This values were observed to have increased to 30, 40 and 50 % in E, A and C respectively at 4 WAI. In addition, 40 % of the tubes were infected when dipped for 2 hours in treatment B(10 g/l Cibaplus + 0.4g/l Amoxycillin) under the periods. The lowest effective mixtures were those of treatments B (90 %) and D(10 g/l Cibaplus + 0.2 g/l Streptomycin + 0.12 g/l Penicillin)(70 %) when dipped overnight together with A (70 and 90 %), C (100 %), D (60 and 80 %) and E (80 and 100 %) when dipped for 2 hours at both periods of observation (Figure1 and Plate 1).

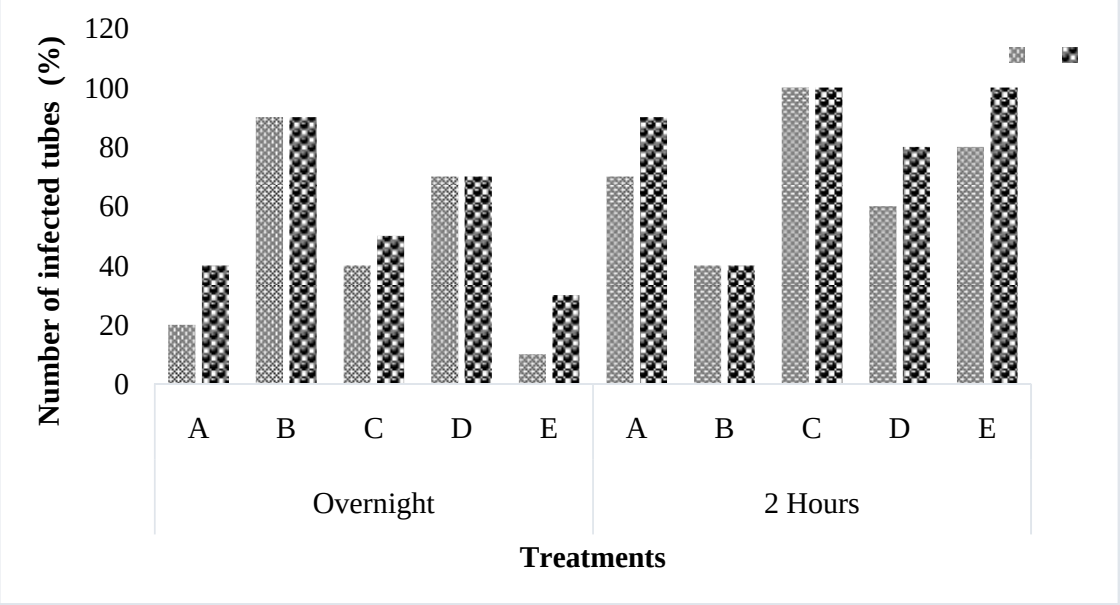


Figure 1: Effect of different fungicide mixtures on microbial growth prevention on in-vitro culture of *V. paradoxa*

A (10 g/l Z-force + 0.4g/l Amoxycillin); **B** (10 g/l Cibaplus + 0.4g/l Amoxycillin); **C** (10 g/l Z-force + 0.2g/l Streptomycin + 0.12 g/l Penicillin); **D** (10 g/l Cibaplus + 0.2 g/l Streptomycin + 0.12 g/l Penicillin); **E** (5 g/l Z-force + 5 g/l Cibaplus + 0.4 g/l Ciproxamed)

Effect of Fungicide mixtures on explants Regeneration

The ability of in-vitro propagated shoot tips of *V. paradoxa* to grow after been treated with different fungicide mixtures was shown in Figure 2. The highest level of regenerative ability (100 %) observed was from explants treated with mixture E when soaked overnight at1 WAI which latter reduced to 60 % at 4 WAI. Whereas, explants subjected to treatment C at 2 hours had zero survival ability as all them were observed with browning tips and heavy contaminations under both periods. Explants from other treatments showed drastic reduction in their regenerative ability from 60 %, 60 % and 70 % to 10 %, 50 % and 20 % in treatments B, C and D when dipped overnight. Treatments A, B and E were reduced to zero from 40, 60 and 20 % accordingly, while it reduced to 10 from 40 % in treatment D when dipped for 2 hours.

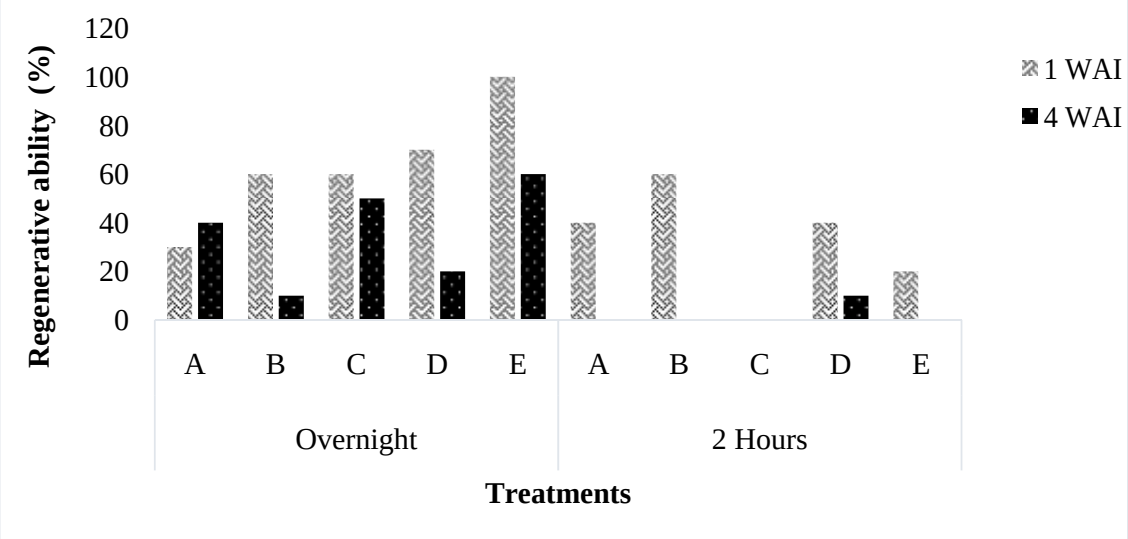


Figure 2: Effect of fungicide mixtures on *V. paradoxa* explants regeneration.

A (10 g/l Z-force + 0.4g/l Amoxycillin); **B** (10 g/l Cibaplus + 0.4g/l Amoxycillin); **C** (10 g/l Z-force + 0.2g/l Streptomycin + 0.12 g/l Penicillin); **D** (10 g/l Cibaplus + 0.2 g/l Streptomycin + 0.12 g/l Penicillin); **E** (5 g/l Z-force + 5 g/l Cibaplus + 0.4 g/l Ciproxamed)

The lethal effect of the fungicide mixtures used was presented in Figure 3 and Plate 1. Dipping the explants in treatments C and E for 2 hours showed no toxicity but heavily contaminated at 1st and 4th WAI. Whereas, treating the explants for 2 hours in treatment A showed the most toxic effects at both periods of investigation; 70 % of the inoculated explants were affected at 4 WAI. The minimum lethal effects (10 %) was obtained from treatment A, D and E when dipped overnight similar to B and D when dipped at 2 hours. Treatment C showed mild toxicity on the explants as 40 % of their plantlets were affected when treated overnight.

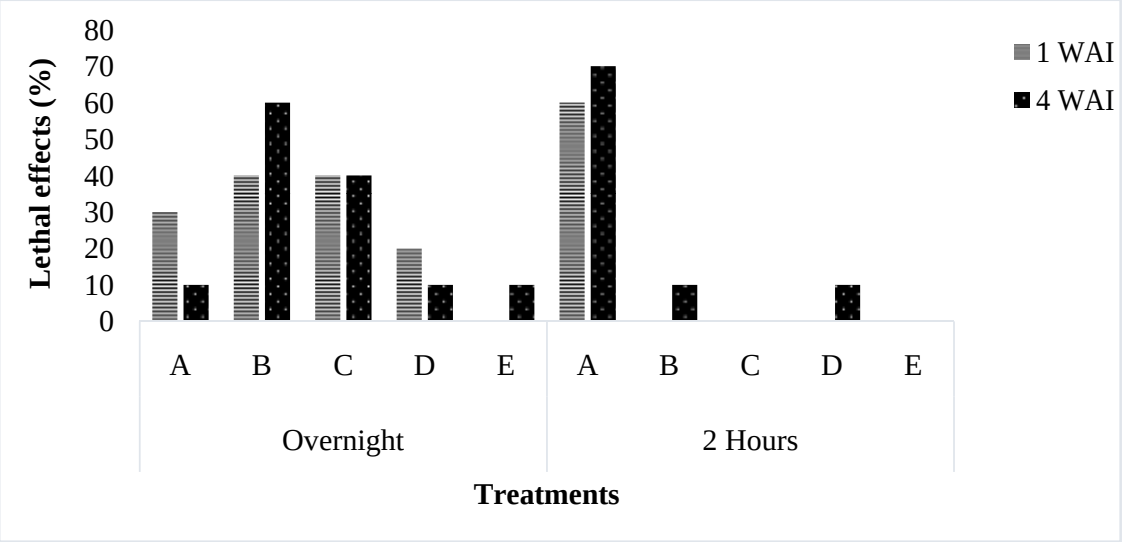


Figure 3: Lethal effect of fungicide mixtures on explants of *V. paradoxa*.

A (10 g/l Z-force + 0.4g/l Amoxycillin); **B** (10 g/l Cibaplus + 0.4g/l Amoxycillin); **C** (10 g/l Z-force + 0.2g/l Streptomycin + 0.12 g/l Penicillin); **D** (10 g/l Cibaplus + 0.2 g/l Streptomycin + 0.12 g/l Penicillin); **E** (5 g/l Z-force + 5 g/l Cibaplus + 0.4 g/l Ciproxamed)

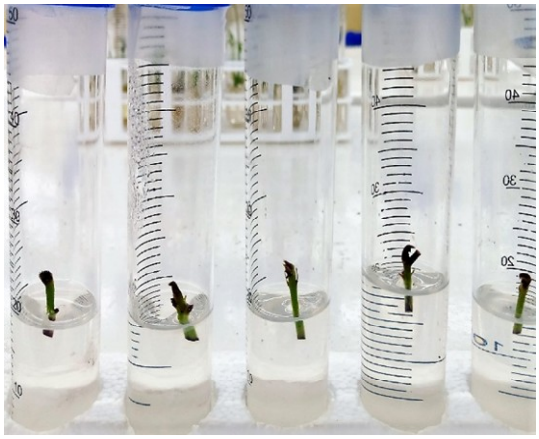
A1

B1

C1

D1

E1



B2

D2

Plate 1. Microbial free cultures of *V. paradoxa* at 4 Weeks after inoculation.

Overnight soaking: A1: (10 g/l Z-force + 0.4g/l Amoxycillin), B1: (10 g/l Cibaplus + 0.4g/l Amoxycillin), C1: (10 g/l Z-force + 0.2g/l Streptomycin + 0.12 g/l Penicillin), D1: (10 g/l Cibaplus + 0.2 g/l Streptomycin + 0.12 g/l Penicillin), E1: (5 g/l Z-force + 5 g/l Cibaplus + 0.4 g/l Ciproxamed). **2 hours soaking:** B2: (10 g/l Cibaplus + 0.4g/l Amoxycillin), D2**:**(10 g/l Cibaplus + 0.2 g/l Streptomycin + 0.12 g/l Penicillin)

In relation to time of dipping, the average number of infected tubes (46 and 56 %) was lower while the average ability of the explants to regenerate (64 and 36 %) was higher when dipped overnight compared with 2 hours of dipping 70 and 80 %, 32 and 2 % respectively (Figure 4). Nonetheless, 26 percent of the explants inoculated were averagely killed by the fungicide mixtures as a result of overnight dipping compared to 18 percent when dipped for 2 hours at both periods of observations.

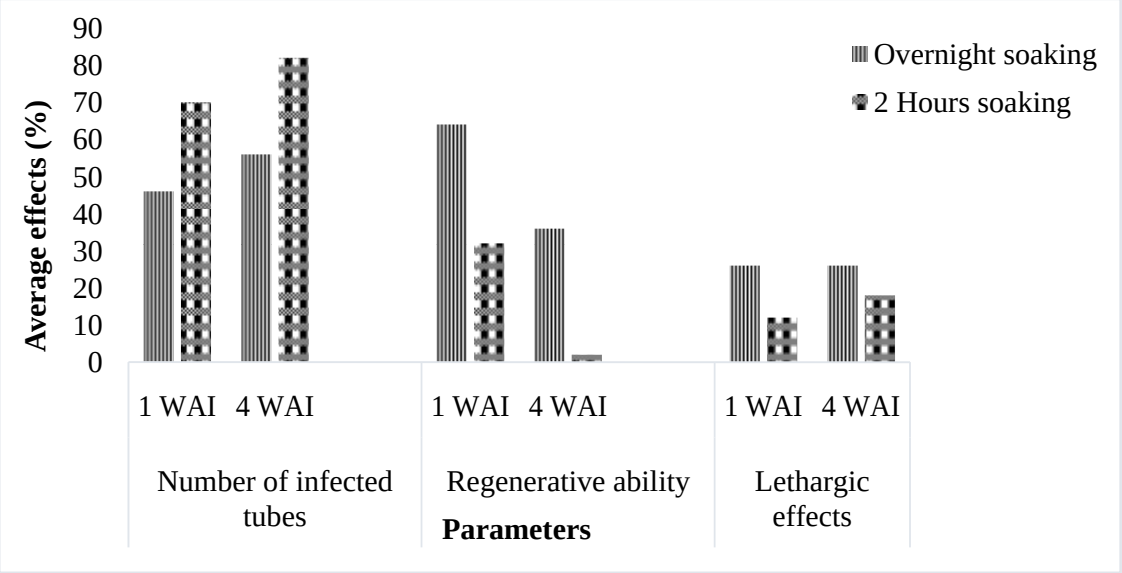


Figure 4: Effect of dipping time on microbial growth prevention and regeneration of *V. paradoxa* explants.

Discussions

Microbial contamination is often a major problem hindering the successful culturing of explants collected from the wild (Singh *et al*., 2011). Evaluation of the effect of some available fungicides and antibiotics on the *in-vitro* culture of *V. paradoxa* explants collected from seedlings under management was the basis of this work. The highest microbial growth prevention observed from treatment E (5 g/l Z-force + 5 g/l Cibaplus + 0.4 g/l Ciproxamed) when explants were dipped overnight could be attributed to the combined effect of the active ingredients (Z-Force: Mancozeb 80% and Cibaplus: Imidacloprid 10% + Metalaxyl 10% + Carbendazim 10%) contained in the fungicide used (Table 1 and Figure 1). Carbendazim is a broad systemic fungicide with protective and curative action while Mancozeb is a contact multi site protective fungicide. The combined use of the two would have ensured double protection from inside and from outside compared with their individual use at double concentration dose.

Across all treatments, observation at first Week after Inoculation (WAI) revealed that the number of infected tubes were as low as

10 % in treatment E but latter increased with time to as much as 100 % in treatment C at 4 WAI. These phenomena could be due to development of resistance by the endogenous contaminants thereby leading to reduction in the effectiveness of the fungicide mixtures and increased infected tubes. Simon *et al*., (2012) stated that discharge of cell sap (nutrients) could stimulates an outgrowth of endogenous fungi that might become pathogenic to the explants under in vitro conditions.

The high phyto-tocixity observed on the explants immersed in Z-force with either 0.4g/l Amoxycillin or0.2g/l Streptomycin + 0.12 g/l Penicillin (treatment A and C) overnight could be due to high concentration of their active ingredients and the contact or exposure time. Whereas, the low survival of most explants exposed to all the treatments for a period of 2 hours was attributed to early onset of high contamination which could be due to inherent pathogens in the explants or contamination from the environment as reported in the findings of Ahmed *et al*., (2010). This result was also similar to the findings of Ankita and Handique, (2010) when the combination of fungicide (Carbendazim) and antibiotics (Amoxicillin) was used to control contamination on the medicinal plant; *Andrographis paniculata* obtained from the wild. They observed that high concentration of the antibiotics led to the death of the explants while amoxicillin at 0.2 % and carbendazim at 0.1 % for 8 minutes followed by normal surface sterilization was effective in controlling bacteria contamination and fungi growth up to 98% success.

In all, the overall number of infected explants were lower in explants dipped overnight in the fungicide mixtures compared to the 2 hours dipping (Figure 4). This showed that overnight dipping allowed the treatment to penetrate the cell of the explants and inhibit growth of micro organisms at that prolong period of contact. This effect and observation is likened to the findings of (Webster *et al*., 2003) who did similar experiment using a combination of 5g/L benlate with 10% bleach solution adjusted to pH 7 to treat wild explants (leaf and nodal segments) of *Petiveria alliacia* and Ackee (*Blighia sapida*) overnight. They stated that extending the sterilization time overnight compared with one and four hours exposure led to reductions in both fungal (decreased from 46% to 27%) and bacterial contamination (decreased from 49% to 12%).

The use of 0.4 g/l Ciproxamed in Treatment E provided effective control for bacterial contaminants compared with 0.4 g/l Amoxicillin, 0.2g/l Streptomycin and 0.12 g/l Penicillin in this experiment. This result was different from the report of Ankita and Handique, (2010) where bacterial contamination free culture of *Andrographis paniculata* was obtained using 0.2 g/l Amoxycillin. Ankita and Handique further stated that the concentration and choice of antibiotic should be such that the desired effect of eliminating contaminants should not threaten the survival of the plant in terms of adverse side effects such as necrosis and phytotoxicity. Antibiotics such as Amoxycillin, tetracycline, ampicillin, cefataxamine and chloramphenicol have been reported to have inhibitory effect on bacterial contaminants without phyto-toxicity (Herman, 1996). Hence the choice of antibiotics used for this study and results obtained.

Conclusion

Successful microbial-free culture initiation in plant tissue culture is a prerequisite to achieving tangible results in the subsequent steps. The present study has proved the effectiveness of some available fungicides and bactericides on microbial growth prevention during inoculation of externally

collected explants of *V. paradoxa*. Combination of Z-force/Cibaplus/Ciproxamed at 5/5/0.4 g/l gave highest level of contamination prevention, explants regenerative ability and minimal toxic effect on explants when dipped overnight. Hence, the combination is recommended for sterilization of *V. paradoxa* explants during *in-vitro* propagation.

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