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Effects of priming and inoculation media on *in vitro* germination of *Zanthoxylum zanthoxyloides* (Lam.) seed

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ABSTRACT

In vitro germination of *Z. zanthoxyloides* seeds were investigated. Seeds (240) were collected from the wild. One hundred and twenty seeds were primed in de-ionised water for 24 hours, while the rest served as control. Then, seeds were sterilised using standard procedure and inoculated on combinations of either Woody Plant Medium (WPM) or Murashige and Skoog (MS), with Benzyl Amino Purine (BAP), Naphthalene Acetic Acid (NAA), Indole Butyric Acid (IBA), 2,4 Dichlorophenoxy acetic acid(2,4-D) and Kinetin (KIN). Treatment combinations plus 1.0 mg/l 2,4-D were WPM+2 mg/L BAP+0.5mg/L NAA+10mg/L IBA(M_1), WPM+5mg/L BAP+5mg/L NAA(M_2), WPM+2mg/L BAP+5 mg/L KIN+10mg/L IBA(M_3), WPM+5mg/L BAP+5mg/L KIN+10mg/L IBA (M_4), WPM (M_5), MS+2mg/L BAP+0.5mg/L NAA+10mg/L IBA(M_6), MS+5mg/L BAP+5mg/L NAA(M_7), MS+2mg/L BAP+5mg/L KIN+10mg/L IBA(M_8), MS+5mg/L BAP+5mg/L KIN+10mg/L IBA(M_9), MS (M_{10}). A 2x10 factorial in completely randomised design was used. Number of days to germination (NDG), percentage seed germination (PSG) and root length (RL) were recorded and analysed using descriptive statistics and ANOVA at $\alpha_{0.05}$. Effects of priming, inoculation media and their interactions were significant for the measured variables. The NDG ranged from 3.75±2.5 days (M_1) to 25.5±9.0 days (M_{10}) and 5.50±4.00 days (M_{10}) to 37.0±14.5 days (M_4); PSG ranged from 6.25±5.50 % (M_2) to 68.8±23.9 % (M_{10}) and 6.25±5.50 % (M_2 and M_{10}) to 50.0±28.9 % (M_3); RL ranged from 0.25±0.19cm (M_2) to 0.88±0.48 cm (M_9) and 0.10±0.04 cm (M_2) to 0.88±0.26 cm (M_9) in primed and unprimed seeds. Priming and growth media improved germination. Modified MS and WPM Media are recommended as germination growth media for *Zanthoxylum zanthoxyloides*.

Keywords: Plant growth regulator, *in vitro*, seed germination, priming

INTRODUCTION

Zanthoxylum zanthoxyloides species was formerly known under generic name Fagara Linn and it was recent chemosystematic considerations that led to the 'sinking' of Fagara. This species belongs to the sub-family Rutoideae, tribe *zanthoxyleae* in the family Rutaceae (Olatunji, 1983). It is a small tree of up to 18m high and 13cm girth. A major characteristic is that the trunks, branches, branchlets, leaf stalks and inflorescence axes of all these species are covered by prickles or what others described as spines (Waterman, 1986) (Figure 1). This makes the *Zanthoxylum* species a big nuisance to farmers who always mark them for destruction when sighted on farms and vegetations. It is primarily a plant of the forest vegetation and found frequently in the southern parts of Nigeria. Economically, the timber of *Z. zanthoxyloides* is used in house and boat-building, decorative panelling, joinery,

making of talking drums and in the pulp and paper industry (Adeniyi *et al.*, 2010).

The root, bark, and leaves of the species are used in various medicinal preparations for curing sickle cell anaemia, stomach ache, tooth ache, coughs, urinary and veneral diseases, leprous ulcerations, rheumatism and lumbago (Olatunji, 1983; Quattara *et al.*, 2004; Adesina, 2005; Odugbemi, 2006). Adeniyi *et al.* (2010) also investigated the antimicrobial activities of *Z. zanthoxyloides* and found its crude extracts inhibited the growth of fungi and bacterial. They further reported that the microbial growth inhibition property is an indication that the plant can be used as a source for antimicrobial agent in the formulation of toothpaste, thus justifying the use of the plant locally as chewing stick. Of great interest too is the isolation and identification of antifungal and antioxidant compounds from the root bark of *Z. zanthoxyloides* (Chaaib *et al.*, 2003). Studies have shown that the solvent extract of *Zanthoxylum zanthoxyloides* is a good repellant of stored grains insect pests. For instance, Owusi *et al.* (2007) and Udo (2011) revealed that this species gave a hundred percent (100%) protection to maize and cowpea against damage by *Sitophilus zeamais* and *Callosobruchus maculates* respectively, while causing complete inhibition of F_1 progeny production and egg development within grains. The study species is a wild economic and medicinal plant that is threatened and endangered by genetic erosion (IUCN, 2011). Currently, the plant is facing a threat of extinction arisen from destructive harvesting of plant parts for medicinal use, and deforestation. Also, it is a tree species that requires many years to reach reproductive maturity However, conventional/traditional methods of regeneration through seed, stem and stem cuttings are too slow due to low viability, low germination rate and delayed rooting of seedling. Therefore, in order to produce uniformly growing seedlings as well as disease-free species within a small space, in a short time, *in vitro* germination is the best alternative (Ng, 2000).This paper seeks to propagate *Zanthoxylum zanthoxyloides* through *in vitro* germination, in order to determine the effect of priming and growth media on *in vitro* seed germination because people's attention is now shifting to phytomedicine for the treatment of ailments such as sickle cell anemia, broncho-pneumonia because plants are relatively safer, more affordable, and accessible without side effects as compared to orthodox medicine.

Figure 1*:* Mature tree of *Zanthoxylum zanthoxyloides*

MATERIALS AND METHODS

The study was carried out in the Tissue Culture Laboratory of the Department of Agronomy, University of Ibadan, Nigeria. The viability of collected seeds from the wild was determined by flotation technique. Seeds were poured into a basin filled with tap water for a few seconds. Seeds that settled at the bottom were assumed to be viable and used for further experiments. All glass wares, including beaker, tools like forceps, scrapper were wrapped with aluminum foil and autoclaved at 121^0C for 20 minutes (sterilization). Two hundred and forty viable seeds (240) were washed thoroughly under running tap water to remove dirt and dust particles from the seed coat. One hundred and twenty seeds of *Zanthoxylum* were primed in de-ionised water for 24 hours while the rest served as control. After priming, seeds were surface sterilized in 70% ethanol for 2 minutes, decanted and transferred to 50% Clorox (Sodium hypochlorite solution) for additional 5 minutes, and then rinsed thoroughly with sterile distilled water, prior to *in vitro* culture on nutrient media with plant growth regulator (Table 1). Two media types: Murashige and Skoog, MS (1962) and Woody Plant Medium, WPM (McCown and Sellmer, 1987) were supplemented with plant growth regulators: Benzyl amino purine (BAP), kinetin (KIN), Naphthalene acetic acid (NAA), indole-3 butyric acid (IBA) plus 1.0 mg/l 2, 4-D into the treatment media combinations. Stock solutions of the media were prepared and stored in the refrigerator with Stock solutions of five (5) growth hormones used and stored in the refrigerator, 100mls of each was prepared. Appropriate quantities of the stock solutions of each media combination with growth hormones and 30 g/l sucrose was added into each medium which was adjusted to a pH of 5.7 with 0.1 or 1.0M NaoH or HCl before the addition of 7 g/l Agar. The media were dispensed into sterile bottles at a rate of 28 ml / bottle and sterilised in an autoclave for 20 minutes at 121°C. The media were allowed to cool before the addition of sterile gentamycin at 2 mg/l for the control of endogenous bacterial contaminations in the Lamina flow hood. All plant growth regulators were from Sigma Aldrich Co., St Louis, MO, USA.

Inoculation and culture conditions

The sterilised seeds (both primed and unprimed) of the species were cultured on Murashige and Skoog and Woody Plant Medium containing ten treatments (10), each with three (3) seed explants per bottle with four replicates each. The cultures were incubated in a growth room at 26 $\pm$ 2^0C under a 16h photoperiod with cool-white fluorescent light and later arranged on the shelves in the growth room. Treatments were arranged as 2 x 10 factorial experiment in a Completely Randomised Design (CRD). The factors are: 2 for priming and unpriming and 10 for culture variant/treatment (media). The experiment was observed fortnightly to determine the Number of Days to Germination (NDG) counted in days; Percentage Seed Germination (PSG) and Root Length (RL) were recorded after 4 weeks of culture using ruler in centimeter. Percentage seed germination (PSG) was recorded after 4 weeks of culture and was calculated using formula:

$$PSG = \frac{\text{Number of germinated seeds}}{\text{Total number of seed inoculated under each medium}} \times \frac{100}{1}$$

Data were analysed using descriptive statistics and analysis of variance (ANOVA) at $\alpha_{0.05}$ and the observed means of characters were subjected to Duncan Multiple Range Test (DMRT) at $\alpha_{0.05}$ to show mean separation.

Table 1: Nutrient Media preparation for *Z. zanthoxyloides*

Treatment code	Media
M_1	WPM + 2mg/l BAP + 0.5 mg/l NAA + 10 mg/l IBA + 1 mg/l 2,4D
M_2	WPM + 5 mg/l BAP + 5 mg/l NAA + 1 mg/l 2, 4D
M_3	WPM + 2 mg/l BAP + 5 mg/l KIN + 1 mg/l 2,4D
M_4	WPM + 5 mg/l BAP + 5 mg/l KIN + 10 mg/l IBA + 1 mg/l 2,4D
M_5	WPM + 1 mg/l 2,4D
M_6	MS + 2 mg/l BAP + 0.5 mg/l NAA + 10 mg/l IBA + 1 mg/l 2,4D
M_7	MS + 5 mg/l BAP + 5 mg/l NAA + 1 mg/l 2,4D
M_8	MS + 2 mg/l BAP + 5 mg/l KIN + 10 mg/l IBA + 1 mg/l 2,4D
M_9	MS + 5 mg/l BAP + 5 mg/l KIN + 10 mg/l IBA + 1 mg/l 2,4D
M_{10}	MS + 1 mg/l 2,4D

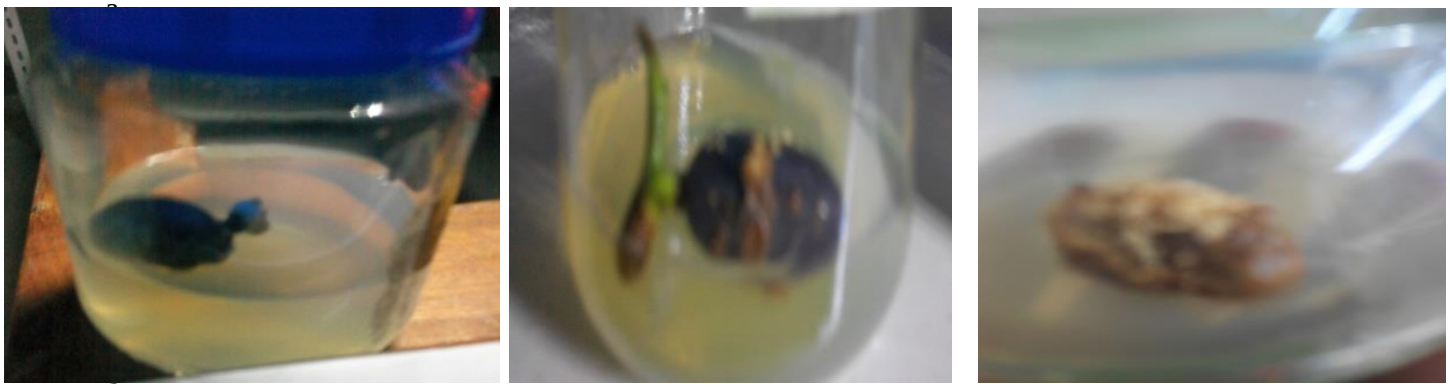
RESULTS

Number of days to germination (NDG)

Germination commenced on the 4th day in primed seeds of *Z. zanthoxyloides* inoculated in M_1 while it took 37.0 days for unprimed seeds to germinate in M_4. The *mean* NDG of *Z. zanthoxyloides* ranged from 3.75±2.5 days (M_1) to 25.5±9.0 days (M_{10}) and 5.50±4.00 days (M_{10}) to 37.0±14.5 days (M_4) in primed and unprimed seeds, respectively. Primed seeds did not germinate (NDG 0.00±0.00 days) in M_3 and M_4 while unprimed seeds did not germinate (NDG 0.00±0.00 days) in M_5 (Table 2).

Table 2: Mean of Number of days to germination for *Z. zanthoxyloides*

Media	Number of days to germination (days)	
	Primed seeds	**Unprimed seeds**
M_1	3.75±2.50	20.00±0.00
M_2	6.75±5.50	8.25±7.50
M_3	0.00±0.00	20.25±7.76
M_4	0.00±0.00	37.00±14.45
M_5	17.25±11.50	0.00±0.00
M_6	6.00±5.93	15.00±14.32
M_7	20.50±14.98	10.25±9.89
M_8	13.50±12.6	9.50±7.97
M_9	23.00±0.00	19.00±0.00
M_{10}	25.50±9.00	5.50±4.00



a **b** **c**

Figure 2: *In vitro* seed germination of *Z. zanthoxyloides*

a: Emergence of radicle

b: Rooted *Z. zanthoxyloides*

c: Callus formation on modified media with 2,4-D

Percentage seed germination (PSG)

The highest Percentage seed germination of 68.8 % was recorded in primed seeds inoculated on M_{10} while the lowest value of 6.25% was obtained for both primed and unprimed seeds inoculated on M_2 as well as unprimed seeds in M_{10}. The PSG ranged from 6.25±5.50 % (M_2) to 68.8±23.9 % (M_{10}) and 6.25±5.50 % (M_2 and M_{10}) to 50.0±28.9 % (M_3) in primed and unprimed seeds respectively after the addition of 2,4-D. The lowest PSG (0.00±0.00 %) was observed in (M_3, M_4) and M_5 in primed and unprimed seeds respectively (Table 3)

Table 3: Mean of Percentage Seed Germination (PSG) in *Z. zanthoxyloides*

Media	Percentage seed germination (%)	
	Primed seeds	**Unprimed seeds**
M_1	18.75±12.5	25.00±0.00
M_2	6.25±5.50	6.25±5.50
M_3	0.00±0.00	50.00±28.9
M_4	0.00±0.00	25.00±0.00
M_5	25.00±0.00	0.00±0.00
M_6	25.00±0.00	18.75±16.9
M_7	18.75±12.5	12.50±11.4
M_8	25.00±28.9	31.25±23.9
M_9	50.00±20.4	37.50±25.0
M_{10}	68.75±23.9	6.25±5.50

Root length (RL)

The highest mean root length of 0.88±0.26 cm was obtained in seedlings of primed seeds in Media M_9 while the lowest mean value (0.10±0.04cm) was in M_2 for unprimed seeds. There was neither germination nor root formation in media M_3, M_4 and M_5 for both primed and unprimed seeds of Z. *zanthoxyloides.* The RL ranged from 0.25±0.19cm (M_2) to 0.88±0.48 cm (M_9), 0.10±0.04 cm (M_2) to 0.88±0.26 cm (M_9) after the addition of 2,4-D in primed and unprimed seeds, respectively (Table 4).

Table 4: Mean Root length in *Z. zanthoxyloides*

Media	Primed seeds			Mean Root Length (cm)	Unprimed seeds			Mean Root Length (cm)
	Wk 2	Wk 4	Wk 6		Wk 2	Wk 4	Wk 6	
M_1	0.10±0.08	0.35±0.33	0.40±0.32	0.28	0.10±0.08	0.28±0.21	0.45±0.30	0.28
M_2	0.13±0.11	0.13±0.11	0.25±0.19	0.17	0.05±0.10	0.08±0.15	0.10±0.04	0.08
M_3	0.00±0.00	0.00±0.00	0.00±0.00	0.00	0.15±0.06	0.58±0.15	0.58±0.15	0.44
M_4	0.00±0.00	0.00±0.00	0.00±0.00	0.00	0.15±0.14	0.20±0.28	0.35±0.19	0.23
M_5	0.10±0.08	0.38±0.35	0.45±0.42	0.31	0.00±0.00	0.00±0.00	0.00±0.00	0.00
M_6	0.15±0.14	0.38±0.28	0.50±0.41	0.34	0.25±0.19	0.25±0.19	0.25±0.19	0.25
M_7	0.05±0.04	0.20±0.12	0.35±0.24	0.20	0.15±0.14	0.20±0.15	0.25±0.19	0.20
M_8	0.05±0.04	0.15±0.10	0.38±0.28	0.19	0.05±0.06	0.15±0.17	0.25±0.19	0.15
M_9	0.50±0.00	0.60±0.00	0.88±0.48	0.66	0.58±0.25	0.68±0.40	0.88±0.26	0.71
M_{10}	0.15±0.06	0.43±0.10	0.75±0.29	0.44	0.13±0.25	0.13±0.25	0.13±0.15	0.13

DISCUSSION

Early germination in woody plant medium (WPM+2mg/lBAP+0.5mg/lNAA+10 mg/l IBA) could be traced to the concentration and combination of growth hormones in the culture. This is similar to Su *et al.* (2011) who found that low concentration of auxin combined with cytokinin might aid shoot initiation. Hence, the ratio of cytokinin to auxin is a critical determinant of organogenesis in plant tissue culture (Xu *et al*., 2008).On the other hand, late germination is due to high exogenous application of growth hormones which delayed the growth of the plant because higher application inhibits growth. Similar observation was made by Buising *et al*. (1994), Strosse *et al*.(2004) and Jafari *et al.* (2011) implying that higher application of cytokinin tends to have adverse effect on the multiplication rate and morphology of the explant. The highest percentage germination observed in MS media supplemented with 5BAP +5KIN+10IBA could be due to media composition. This result is similar to the report of Elkonin and Pakhomova (2000), who reported that media composition seems to be the key factor affecting morphogenesis in cells grown *in vitro*. Lowest percentage germination in WPM media supplemented with 5BAP+ 5NAA could be traced to the combination of hormones and the type of media which is similar to McCrown and Sellmer (1987) who reported that some species show preference for a specific medium for explant establishment and growth. Also, tissue growth and the quality of morphogenetic responses are strongly influenced by the type and concentration of nutrients present in the culture media (Niedz and Evens, 2007). The highest

percentage seed germination in pure MS media could be attributed to the effect of auxins in the breaking of seed dormancy. Wang *et al.* (2005) reported the positive effect of 2,4-D on seed germination of *Rhodiola rosea.* The MS media supplemented with high concentration of growth hormones had the highest effect on the root of zanthoxylum and this could be as a result of increase in BAP concentration leading to increased rooting. This result is contrary to Asghari *et al.* (2012) who reported that increasing BAP concentration led to decreased rooting in *Ocimum basilicum*. On the other hand, shortest root length was influenced by WPM supplemented with high concentration of growth hormones in 5mg/l BAP+ 5mg/l NAA. This is due to type of media used in the study. This result is similar to Niedz and Evens (2007) who reported that tissue growth and the quality of morphogenetic responses like rooting are strongly influenced by the type and concentration of nutrients present in the culture media. In this study, addition of 2,4-D induced callus formation which was in conformity with the work of Sambe *et al.* (2010) who reported the callus was induced by 2,4-D and not by NAA on the seed germination and *in vitro* regeneration of *Parkia biglobosa*.

CONCLUSION

Due to the indiscriminate cutting of trees plus the recalcitrant nature of seeds of tropical species, it is important to devise faster methods of regeneration. The study has shown that the seeds of *Z. zanthoxyloides* could germinate faster by priming with water. The study has also provided the best growth media for the species. From the results of this study, it is recommended that the seeds of *Z. zanthoxyloides* should be primed in water for 24 hours before sowing *in vitro*, the seeds should be treated with1.0 mg/L of 2,4 dichlorophenoxy acetic acid and inoculated in Woody Plant Medium (WPM),supplemented with 2mg/lBAP+0.5mg/lNAA+10mg/lIBA for early germination.

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