E-ISSN: 2346-7290

In vitro seed germination of *Pycnanthus angolensis* (Welw.)

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ABSTRACT

The effects of priming and growth media on *in vitro* germination of *P. angolensis* seeds were investigated. Seeds (240) of *P. angolensis* were collected from the wild. One hundred and twenty seeds of the species were primed in de-ionised water for 24 hours, while the rest served as control. Then, seeds were sterilized using standard procedure followed by inoculation 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) and Kinetin (KIN). Treatment combinations were WPM+2mg/L BAP+0.5mg/L NAA+10mg/L IBA(M_1), WPM+5mg/L BAP+5mg/L NAA(M_2), WPM+2mg/L BAP+5mg/L KIN+10mg/L IBA(M_3), WPM+5mg/L BAP+5mg/L KIN+10mg/L IBA (M_4), WPM only(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 only (M_{10}). A 2x10 factorial experiment in completely randomised design was used. Number of days to germination (NDG), percentage seed germination (PSG) and root length (RL) were measured 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 of *P. angolensis* ranged from 14.0±10.3days(M_2) to 34.8±13.9days(M_1) and 19.0±12.6days(M_6) to 42.0±6.9days(M_1), PSG ranged from 18.8±13.9(M_2) to 62.5±43.3(M_6) and 31.3±27.5%(M_6) to 56.3±12.5%(M_1), RL ranged from 0.50±0.38cm(M_8) to 1.88±1.32cm(M_{10}) and 1.13±1.02cm(M_6) to 1.80±1.40cm(M_8) in primed and unprimed seeds, respectively. Priming and growth media improved germination of *P. angolensis.* The best germination rate in primed seed inoculated on MS with low concentration of BAP and NAA is therefore recommended as growth media for *P. angolensis.*

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

INTRODUCTION

Pycnanthus angolensis Welw. belongs to the nutmeg family, Myristicaceae. It is an evergreen, monoecious or dioecious, medium-sized to large tree up to 25-40m tall. The species occurs in upland and wet evergreen forest and semi- deciduous forest with more than 1600mm rainfall (Orwa *et al.*, 2009). It is especially abundant in old fallows and secondary forest as its rate of natural recruitment after disturbance of the forest is high. In southern Africa, it occurs in riverine and swamp forest, but in West Africa, it does not occur in swamps (Keay, 1987). The bole of the tree is usually straight and cylindrical (Figure 1), branchless for up to 15 – 25m high, up to 120 – 150cm in diameter, usually without buttresses. Its outer bark is grayish brown, with orange-brown exudates. The leaves are

distichously alternate, simple and entire, without stipules. The fruit is ellipsoid to oblong or globose drupe, yellowish orange when ripe (Figure 2). Seeds of *P. angolensis* are recalcitrant with duration of germination from 16 – 36 days after planting (Dike and Aguguom, 2010).The seeds are also aromatic. The plant is commonly called 'Akomu' in Yoruba, 'Oje or Aakwa-mili' or 'Egwunond' in Igbo, 'calabo' in Spanish and 'Akujandi' in Hausa (Rahama, 2011). According to Mapongmentsem (2007), a yellow to reddish brown fat, called 'kombo butter' or 'Angola tallow' is extracted from the seed and is important in west and central Africa for soap making and as illuminant. It is not edible. The seeds somewhat resemble those of nutmeg (*Myristica fragrans* Houtt) and are burnt as candle. The wood is highly valued as fuel and is used to make split planks, known as 'calabot' or caraboard' in the coastal zone of Cameroon (Orwa *et al.*, 2009). Because it is easy to work with, it is used to make shingles, both for roofing and covering the sides of native houses, and planks for doors and window frames. The long straight bole makes it suitable for making canoes. Since the World War II, the wood has become an important timber for plywood core stock, veneer, mouldings, interior trim, interior joinery, furniture components and paper pulp.

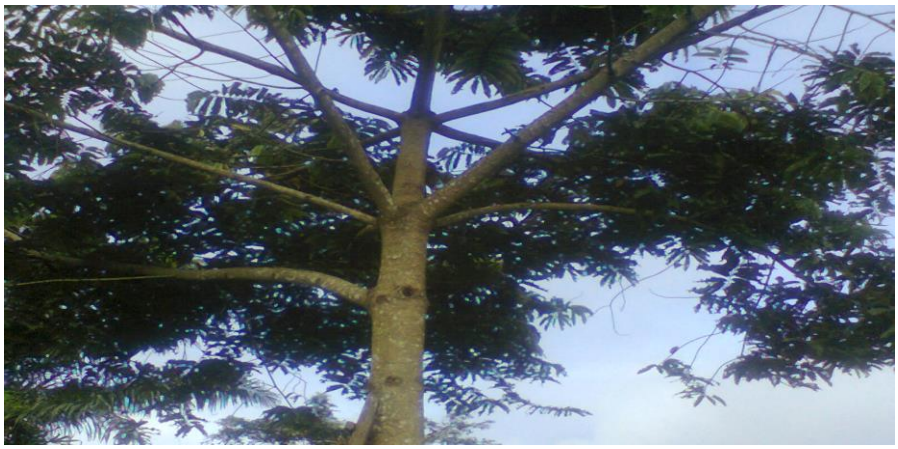


Figure 1: Mature tree of *Pycnanthus angolensis*

Figure 2 Mature fruit of *Pycnanthus angolensis*

Leakey (1999) reported that in agroforestry, *P. angolensis* is planted or retained for shade in coffee, cocoa and banana plantation in the humid lowlands of Cameroon and Uganda. It is planted to provide shade in farms and plantations, and is also suitable for ornamental planting. When the tree is growing wild, it is considered to be a good indicator of soil fertility. The ethno-pharmacological survey of the plant, *Pycnanthus angolensis* was reported by Agyare *et al.* (2009) confirming the potency of the aqueous extracts of the plant for wound healing and established antioxidant activities which is good for stomach ulcer treatment. Also, its anti-adhesive activity against *Helicobacter pylori* on human stomach cells was reported by Burkill (2000) and Ancolio *et al.* (2002). The leaf juice is sucked to cure white tongue thrush and the latex is applied to wounds as an anti-coagulant (Ayensu, 2008). It has also been reported to be useful for the treatment of female sterility, gonorrheal infertility, rhino pharyngeal and broncho-pneumonia and poison antidote (Burkill, 2000). A collyrium of the latex is used for treatment of various eye troubles especially cataract, filariasis of the eye and for schistosomiasis (Ancolio *et al*, 2002). The bark has been used as a poison antidote and a treatment for leprosy, and anaemia. *Pycnanthus angolensis* is also good for the treatment of black tongue, fever, cough, bad breath, anti-billharzia, lumbago, toothache, rube facial and skin infection (Rahaman, 2011). It is also believed to assist lactation (Nsimundele, 2004). *Pycnanthus angolensis* seed has short viability and should be soaked in water for 24 hours to hasten its germination. It is a sun-loving species.

According to Katende, *et al.* (1995), conventional propagation through seeds and stem cuttings did not succeed; and seeds should not be dried but sown as soon as possible. Despite its great contribution to the well being of people, *Pycnanthus angolensis* remains undomesticated on a large scale. The 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 as well as disease-free seedlings within a small space, in a short time, in vitro germination is the best alternative (Ng, 2000). This paper seeks to determine the effect of priming and inoculating media on the *in vitro* propagation of *Pycnanthus angolensis*.

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 was 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). Viable seeds (240 each) of the study species were washed thoroughly under running tap water to remove dirt and dust particles from the seed coat. One hundred and twenty seeds each of Pycnanthus 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) into the treatment media combinations. Stock solutions of the media were prepared and stored in the refrigerator with Stock solutions of four (4) growth hormones used and stored in the refrigerator, 100mls of each were 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 sterilized in an autoclave for 20 min 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 acquired from Sigma Aldrich Co., St Louis, MO, USA.

Inoculation and culture conditions

The sterilized 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^{o}C 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: two for priming and unpriming and 10 for culture variant/treatment (media). The experiment was observed fortnightly for the Number of Days to Germination (NDG) counted in days; Percentage Seed Germination (PSG) and Root Length (RL) measured after 4weeks of culture using ruler in centimeter. Percentage seed germination (PSG) was calculated using formula:

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

Table 1: Treatments used for *in vitro* seed germination

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

RESULTS

Number of days to germination (NDG)

Germination commenced first in primed *P. angolensis* inoculated in M_2 on the 14th day after inoculation while it took 42 days for umprimed seeds to germination in M_1. Germination did not occur in primed seeds inoculated on M_3, M_4 and M_{10}; and unprimed seeds inoculated on M_2, M_3, M_4, M_5, M_7, and M_{10}. The NDG in *P. angolensis* ranged from 14.0±10.3 days (M_2) to 34.8±13.9 days (M_1) and from 19.0±12.6 days (M_6) to 42.0±6.9 days (M_1) in primed and unprimed seeds, respectively. (Table 2 and figure 3).

Percentage seed germination (PSG)

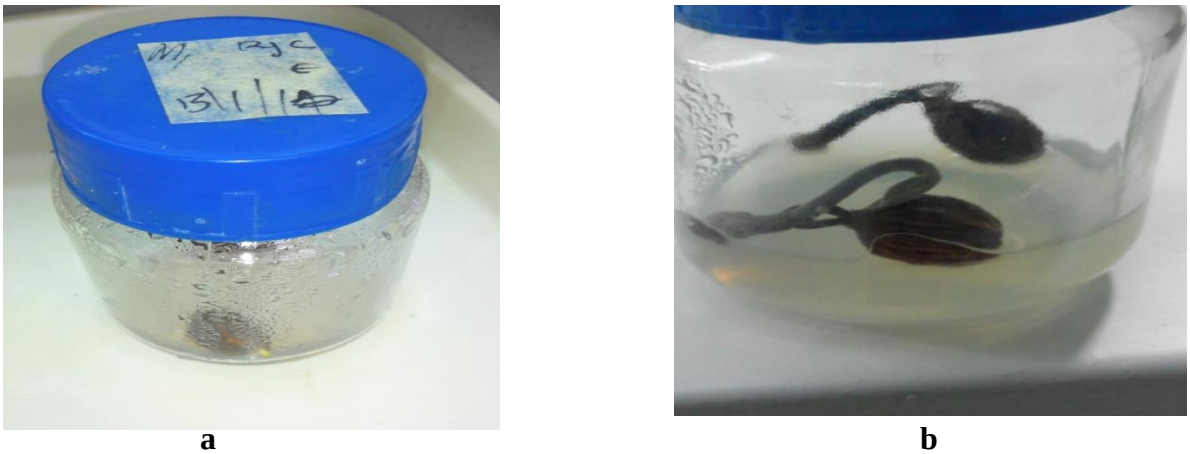
The highest percentage seed germination 62.5% was observed in M_6 for primed seeds while the lowest value of 18. 8% was recorded for primed seeds in M_2. (Table 3).The PSG ranged from 18.8±13.9 (M_2) to 62.5±43.3 (M_6) and 31.3±27.5% (M_6) to 56.3±12.5 % (M_1) in primed and unprimed seeds, respectively. No germination (0.00±0.00) was recorded in M_3, M_4 and M_9; (M_2, M_3, M_4, M_5, M_7, M_9 and M_{10} in primed and unprimed seeds, respectively (Table 3).

Table 2 Mean Number of days to germination in *P. angolensis*

Media	Number of days to germination NDG(days) Primed seeds	Unprimed seeds
M_1	34.75±13.90	42.00±6.90
M_2	14.00±10.30	0.00±0.00
M_3	0.00±0.00	0.00±0.00
M_4	0.00±0.00	0.00±0.00
M_5	17.50±11.70	0.00±0.00
M_6	19.50±13.10	19.00±12.60
M_7	24.00±4.00	0.00±0.00
M_8	23.50±15.70	24.00±16.00
M_9	0.00±0.00	0.00±0.00
M_{10}	17.00±12.50	0.00±0.00

Table 3 Mean of Percentage Seed Germination in *P. angolensis*

Media	Percentage seed germination (%)	
	Primed seeds	unprimed seeds
M_1	56.25±12.50	56.25±12.50
M_2	18.75±13.90	0.00±0.00
M_3	0.00±0.00	0.00±0.00
M_4	0.00±0.00	0.00±0.00
M_5	43.75±31.50	0.00±0.00
M_6	62.50±43.30	31.25±27.50
M_7	50.00±20.40	0.00±0.00
M_8	25.00±20.40	50.00±35.40
M_9	0.00±0.00	0.00±0.00
M_{10}	37.50±25.00	0.00±0.00



a **b**

Figure 3: *In vitro* seed germination of *P. angolensis*

a: Emergence of radicle of *in vitro* seed

b: Rooted *P. angolensis* in cultured media

Root length (RL) of *P. angolensis*

The highest root length 1.88cm was observed in M_{10} (pure MS media) of primed seeds while the lowest root length 0.50 cm was observed in M_8 for primed seeds. The RL ranged from 0.50±0.38 cm (M_8) to 1.88±1.32 cm (M_{10}) and 1.13±1.02 cm (M_6) to 1.80±1.40 cm (M_8) in primed and unprimed seeds, respectively. No rooting (RL =0.00±0.00 cm) was observed in M_3, M_4 and M_7 for primed and unprimed seeds and the same was also observed in M_2, M_5 and M_7 of unprimed seeds. Rooting did not occur at wk2 in M_8 but occurred at wk4 and wk6 (Table 4).

Table 4 Mean of Root length for *P. angolensis*

Media	Primed seeds				Unprimed seeds			
	Wk 2	Wk 4	Wk 6	Mean Root	Wk 2	Wk 4	Wk 6	Mean Root
M_1	0.23±0.21	0.63±0.25	1.25±0.29	0.70	0.73±0.59	1.40±1.01	1.75±1.19	1.29
M_2	0.08±0.06	0.45±0.31	0.63±0.55	0.39	0.00±0.00	0.00±0.00	0.00±0.00	0.00
M_3	0.00±0.00	0.00±0.00	0.00±0.00	0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00
M_4	0.00±0.00	0.00±0.00	0.00±0.00	0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00
M_5	0.28±0.26	0.75±0.61	0.88±0.63	0.64	0.00±0.00	0.00±0.00	0.00±0.00	0.00
M_6	0.63±0.75	1.65±1.12	1.73±1.20	1.34	0.85±0.67	1.13±1.02	1.13±1.02	1.04
M_7	0.63±0.39	1.38±0.48	1.50±1.29	1.17	0.00±0.00	0.00±0.00	0.00±0.00	0.00
M_8	0.00±0.00	0.48±0.33	0.50±0.38	0.00	1.58±1.09	1.60±1.30	1.80±1.40	1.66
M_9	0.00±0.00	0.00±0.00	0.00±0.00	0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00
M_{10}	0.75±0.96	1.43±1.09	1.88±1.32	1.35	0.00±0.00	0.00±0.00	0.00±0.00	0.00

DISCUSSION

There were differences in the NDG between primed and unprimed seeds; also, germination in primed seeds in different media varied significantly. The early number of days to germination in primed seeds of *P. angolensis* could be traced to high concentration and availability of the combined growth hormones. Similar results have been reported by Shivanna *et al.* (2007) for Ber seeds. Media type and the interaction between the media and priming significantly affected percentage of seed germination. The best germination rate was observed in primed seed inoculated on MS with low concentration of BAP and NAA. Germination and germination rate varied with media and hormonal combination and concentration. Mendoza and Kaeppler (2002) reported that the use of auxin in combination with cytokinin, leads to rapid cell division, forming a large number of relatively small and undifferentiated cells. Both NAA and BAP in the medium could have stimulated seed germination in endangered *P. angolensis*. The germination rate observed in *P. angolensis* could be associated with the hydration of seed cotyledon resulting in a high germination rate. Hardness of the seed coat inhibited germination due to insufficient exposure to water, invariably leading to more seeds not germinating in unprimed seed treatment compared to the primed seed treatments. Rooting is an important phase in successful *in vitro* germination of plant species. Without effective rooting system, plant acclimatization will be difficult and rate of

plant propagation may be severely affected (Goncalves *et al.*, 1998). Increasing BAP concentration and NAA led to decrease in the root length of *P. angolensis.* This report is similar to those of Asghari *et al*. (2012) who worked on *Ocimum basilicum* and found that the longest root length was recorded on MS Media combined with 2mg/l BAP + 0.5mg/l NAA + 10 mg/l IBA. Auxins (NAA, IBA) have characteristic feature of promoting cell division, stem elongation and rooting (Razdan, 2003) at the right concentration while the smallest mean root length suggests sub-optimal concentration. The root length of *P. angolensis* varied with the period of assessment (weeks), priming treatment, the type of media as well as the concentration and combination of growth hormones used. The root length of *P. angolensis* increased over the period of assessments and these may be related to good supply and transport of mineral nutrients and plant growth regulators in tissues. The roots on pure MS (no growth hormones) had the longest root length at week 6 which may be due to the effect of endogenous auxins. This result is similar with Sambe *et al.* (2010) who confirmed that rooting in MS medium alone was as a result of high endogenous auxins contents. Elongation of root could also be as a result of priming of the seeds in water for efficient transport of endogenous rooting hormones.

CONCLUSION

Germination of high value medicinal tree species through mass multiplication is *sine qua non* to their domestication and conservation. Present study deals with protocol involved in *in vitro* germination of *P. angolensis* Welw. Results of priming of *P. angolensis* seeds suggested that the seeds germinate better under priming than when sown directly. MS medium supplemented with cytokinin 2.0mg/lBAP) in combination with auxins (0.05mg/lNAA and 10.0mg/lIBA), proved effective. Therefore, priming and growth media improved germination of *P. angolensis.* Seeds of *P. angolensis* must be primed in water 24 hours before sowing to break the seed coat dormancy. The study provided baseline information for further research on *in vitro* propagation of *P. angolensis*

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