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Effect of Imbibition Time on Hormonal Changes of Germinating *Tamarindus indica* and *Prosopis juliflora*

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Abstract. Imbibition time and hormonal changes play a significant role in seed germination. This study, evaluated the effects of some phytohormones; indole acetic acid (IAA), abscisic acid (ABA), gibberellin and cytokinins) and imbibition time (0, 48 and 96 hours) on Tamarindus indica and Prosopis juliflora. High-Performance Liquid Chromatography (HPLC) was used to determine the concentrations of the hormones. Results indicated significantly higher and faster in P. juliflora than T. indica. The germination rate was 4.1 - 68.1% and 4.0 - 61.4%, and model for inhibition time 28.256ln(x) and 25.791ln(x), respectively. Similarly, results also expressed highly significant variable changes in the concentrations of the four studied phytohormones between T. indica (0.491 - 0.705 mg/ml) and P. Juliflora (0.109 - 1.130 mg/ml). The concentrations of IAA and ABA were significantly higher by 60.6% and 77.7% in the seeds of T. indica than P. juliflora, respectively. P. juliflora had 37.6% and 12.5% higher cytokinin and gibberellin than T. indica, respectively. Cytokinin (0.7951 - 1.0939 mg/ml), gibberellins (0.535 - 0.757 mg/ml), IAA (0.363 - 0.419 mg/ml) and ABA (0.250 - 0.335 mg/ml) also varied significantly over the periods. In general, cytokinin and gibberellins increased by 8.1 - 27.3% and 22.9 - 23.0%, while that of IAA and ABA decreased 13.6 - 15.4% and 26.4 - 34.0%, over the imbibitions time of 0-96 hours. In conclusion, higher germination of P. juliflora is attributed to cytokinin and gibberellins, and the lower germination in T. indica to the higher inhibitory effects of IAA and ABA.

Keywords: germination, imbibition, plant hormone, *Prosopis juliflora, Tamarindus indica*

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1. Introduction

Deforestation poses a serious environmental crisis, thus the establishment of tree nurseries and plantations are key strategies for ecosystem restoration, conservation and environmental protection from desertification. In Nigeria, *Tamarindus indica* and *Prosopis juliflora* are very important economic and afforestation tree species, where the latter is used in herbal medicine and the fruit pulp is edible, while the former is very suitable for shelterbelts and windbreaks in

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arid and semi-arid [1] – [5]. Despite their significance, these tree species encounter germination difficulty owing to their hard seeds coats. Their germination involves chains of morphological and physiological processes, in which water imbibition activates complex hormonal interplay in the seed embryos, therefore unravelling these complex processes would provide significant insight into the germination and nursery management of seeds of these tree species.

Botanists and ecologists have extensively investigated pre-treatment methods for breaking seed dormancy and improving seed germination [7] – [13]. Others have also highlighted on phytohormonal changes that occurred in the process of germination in non-dormant seeds, and in some hard seed-coat dormant tree species, *Parkia biglobosa* and *Acacia senegalensis* [14], [5]. In both cases, cytokinin and gibberellin were the main germination inhibitor and growth promoters, respectively [15] – [16]. However, most reports lacked sufficient empirical support for valid comparison among species, owing to the difficulty in generating data points to continuously monitor the rate of hormonal changes during germination, the impetus of which was provided by Koornneef et al. [17] for further studies. In general, six phytohormones namely, abscisic acid (ABA), ethylene, gibberellin, auxin (IAA), cytokinins and brassinosteroids had been strongly implicated in seed dormancy and germination and controlled many physiological and biochemical processes in the plants [18] – [20], [16]. Specifically, amides such as mustard oil, various organic acids, unsaturated lactones, essential oils, alkaloids, phenolics, especially abscisic acid played inhibitory roles [21]. In contrast, nitrate and gibberellin enhance seed germination [22], [24] – [25].

Water uptake is a fundamental requirement in eliciting hormonal activity, and Scientists had observed higher hormonal levels as the result of increased imbibition [23], [26], [17]. The triphasic uptake of water by a dry seed includes a rapid initial imbibition (phase I), plateau phase (phase II) and further increased uptake in the post-germination phase (phase III) [27] – [30]. The foregoing literature, have clearly expressed the need for further quantitative assay of phytohormones changes during early germination phase I – III. Therefore, this study was conceived to further explore the roles of cytokinins, abscisic acid, gibberellin and auxins in seed germination that would involve *in-vitro* monitoring of water imbibition vis-à-vis phytohormones dynamics. This is with the bid to providing supportive empirical backing in explaining the hormonal changes that occur during dormancy and germination and also offer interspecies comparison of differences and/or similarities between *T. indica* and *P. juliflora*. The objectives of the study was to determine the effects of imbibition time on hormone levels in seeds of *T. indica* and *P. juliflora*.

2. Materials and Methods

The hormonal analysis was done at National Agency for Food and Drugs Administration and Control (NAFDAC) Laboratory in Maiduguri, Borno State, Nigeria.

2.1. Sample Collection and Preparation

Fresh samples of matured *Tamarindus indica* and *Prosopis juliflora* seeds were obtained from the mother plants of good phenotype around Lake Chad Research Institute, Maiduguri, Borno State. Seeds were carefully removed from the dry fruits with hand, and then surface-sterilized with 10% hydrogen peroxide. Sound seeds of uniform size were sorted out using simple floatation method (specific gravity grading) described by Suma and Srimathi [31], in which seeds that floated in distilled water using a 750 ml beaker were discarded and those that sunk were used for the study. These were air-dried and then kept in a tight container, at room temperature for subsequent use in the experiment

2.2. Experimental Design and Treatments

A two-factor experiment was conducted using Randomized Complete Block Design (RCBD) with factorial layout (arrangements) of treatments. The factors and treatments were tree species (*Tamarindus indica* and *Prosopis juliflora*) and imbibition time (0, 48 and 96 hours).

2.3. Extraction and Determination of Hormonal Concentration

The seeds of *Tamarindus indica* and *Prosopis juliflora* (approximately 1 g weight) were ground in liquid nitrogen, homogenized and then extracted with 30 ml of 80% cold aqueous methanol. The extract was sonicated using an ultrasonic mixer for 30 minutes then centrifuged at 2000 r/min and 4 °C for 15 minutes and the supernatant was collected. Thereafter, fresh cold 80% methanol was poured into the remnant, extracted three times by the aforementioned methods. The total methanolic extract was dried in a rotary evaporator and dissolved in 10 ml of 100% methanol. Indole-Acetic Acid (IAA), Abscisic acid (ABA), Gibberellin and Cytokinins were measured by the injection of the extract into a reverse-phase High-Performance Liquid Chromatography (HPLC), with a methanol gradient in 0.6% acetic acid. The Standards and Reagents were of analytical grade. Chromatographic column: Hypersil ODS C18 column (150 mm × 4.6 mm, 5 μm); mobile phase: methanol - 0.6% ethanoic acid; gradient elution column temperature: 35°C; sample size: 10 μl; flow rate: 1 ml/min; Ultraviolet detection wavelength of hormones: 254 nm. High-Performance Liquid Chromatography (HPLC) was used to determine the concentrations of some plant hormones as described in Tang et al. [32].

2.4. Preparation of Standard

Precisely 10 mg of cytokinin, gibberellin, abscisic acid and indole acetic acid standards were separately diluted in 50 ml of distilled water. Then 1 ml of diluted standards was each taken and re-dissolved in 1.5 ml of distilled water resulting in a working concentration of 0.34 mg/ml of each of the standards. The final concentration of hormone in the seeds sample was determined according to treatment, using the Beer-Lambert's Law formula:

Working concentration of sample/Working concentration of standard = Peak area of Sample/Peak Area of Standard.

2.5. Data Analysis

Data generated were subjected to statistical analysis, using the software (Staistix Ver. 8.0), analysis of variance (ANOVA) was computed and compared using Least Significant Difference (LSD) at $p<0.05$. Charts were drawn using Microsoft Excel (2003) to depict the pattern and rate of germination in the two tree species, with the coefficient of determination (r2), intercept (constant) and regression coefficient (b-value) as quantitatively measures of differences. In the same vein, the chart for imbibition displayed a) coefficient of determination (r2) as extent (effects) of the relationship between hormones and imbibition time, b) intercept as a measure of initial hormonal level in dormant seed, and c) regression coefficient (b-value) to quantitatively depict the rate of hormonal changes over the germination periods of 0 hours, 48 hours and 96 hours.

3. Results and Discussion

The result on hormone concentration in the two tree species (*T. indica* and *P. juliflora*) and imbibitions time (0, 48, and 96 hours) is shown in Table 1. Results showed that concentrations of the four studied phytohormones differed significantly ($p<0.01$) between the two tree species. In the result, cytokinin, gibberellin, indole acetic acid and abscisic acid concentrations were 0.7056 mg/ml, 0.5988 mg/ml, 0.5719 mg/ml, and 0.4913 mg/ml in *T. indica*, as against 1.1302 mg/ml, 0.6843 mg/ml, 0.2251 mg/ml, and 0.1095 mg/ml in *P. Juliflora*, respectively. Consequently, indole acetic acid and abscisic acid were significantly higher by 60.6% and 77.7% in the seeds of *T. indica* than *P. juliflora*, respectively. Conversely, the seeds of *P. juliflora* had 37.6% and 12.5% higher cytokinin and gibberellin than T. indica, respectively. Concentrations of the four phytohormones also varied significantly ($p<0.01$) over the three imbibition periods (Table 1).

Thus, over 0-96 hours imbibition period, cytokinin, gibberellin, indole acetic acid and abscisic acid concentrations ranged from 0.7951 - 1.0939 mg/ml, 0.5354 - 0.7569 mg/ml, 0.3634 - 0.4194 mg/ml and 0.2501 - 0.3351 mg/ml, respectively. The result shows that cytokinin and gibberellins concentrations were both significantly lower at 0 hour (dormant seed) than at 48 and 96 hours, depicting an increase in concentrations of the two hormones; however, the peak concentration of cytokinin occurred at 48 hours. In contrast, concentrations of IAA and ABA generally decreased over imbibitions time of 0-96 hours. Thus, cytokinin and gibberellin significantly increased by 8.1 - 27.3% and 22.9 - 23.0%, respectively. Conversely, indole acetic acid and abscisic acid decreased by 13.6 - 15.4% and 26.4 - 34.0% at 48 and 96 hours, respectively.

Tabel 1. Comparison of Concentrations of the four Phytohormones between the Two Tree Species and Among Germination Time

	Phytohormones concentration (mg/ml)			
Tree species (A)	**Cytokinin**	**Gibberellins**	**Indole acetic acid**	**Abscisic acid**
Tamarindus indica	0.7056	0.5988	0.5719	0.4913
Prosopis juliflora	1.1302	0.6843	0.2251	0.1095
F-test	**	**	**	**
SE±	0.0045	0.0156	0.0009	0.0004
$LSD_{0.05}$	0.0141	0.0491	0.0028	0.0014
$LSD_{0.01}$	0.0201	0.0699	0.0041	0.0020
Germination time (B)				
0 hrs	0.7951	0.5354	0.4194	0.3351
48 hrs	1.0939	0.6323	0.4127	0.3160
96 hrs	0.8648	0.7569	0.3634	0.2501
F-test	**	**	**	**
SE±	0.0055	0.0191	0.0011	0.0005
$LSD_{0.05}$	0.0173	0.0602	0.0035	0.0017
$LSD_{0.01}$	0.0246	0.0856	0.0050	0.0024
Interaction				
A x B	**	*	**	**

Interaction effect of trees species x imbibition period was also significant ($p<0.05$) for all assessed hormones and results are shown in Table 2. In general, the result consistently expressed significantly higher cytokinin and gibberellin in *P. juliflora* than *T. indica.* In contrast, *T. indica* registered significantly higher indole acetic acid and abscisic acid than in *P. juliflora* at all record periods. Germination is the basic propagation process by which seeds develop into plants after dormancy is broken, with water imbibition resulting in different counteracting changes in the concentrations of phytohormones. The present study compared the rate of germination in two tree species, and hormonal dynamics during the early germination phase, and results generally revealed significant variation in the concentrations, patterns and rates of changes among the four studied hormones, cytokinin, gibberellin, indole acetic acid and abscisic acid in the two tree species over imbibition time.

In general, results indicated significantly higher germination in seeds of *P. juliflora* than *T. indica*; consequently, the germination rate was also higher in *P. juliflora* than *T. indica*. Furthermore, results expressed that the concentrations of hormones differed according to species; consequently, indole acetic acid and abscisic acid were comparably higher in *T. indica*, while cytokinin and gibberellin were higher in *P. juliflora.* This is in line with Graeber et al. [16] that abscisic acid (ABA), ethylene, gibberellin, auxin (IAA), cytokinins, and brassinosteroids control many physiological and biochemical processes in the plants. However, the most important plant hormones for seed germination are abscisic acid and gibberellins, which have inhibitory and stimulatory effects on seed germination, respectively. In the present

study, the inverse effects of these two phytohormones on germination could imply higher germination inhibitory effects in *T. indica*, in contrast to higher stimulatory effects in *P. juliflora*. This concurs with the findings of Achard et al. [33] that levels of abscisic acid usually drops during germination, while that of gibberellin and cytokinin increases, however, these hormones interact, and abscisic acid can be reversed by the action of cytokinin. On the other hand, nitrate and gibberellins enhance seed germination, nitrate act as a source of Nitrogen and a seed germination enhancer, and gibberellin enhances seed germination by the activation of catabolizing enzymes and inhibition of the related biosynthesis pathways, which also decreases ABA amounts [22] – [25].

Table 2. Response of *Tamarindus indica* and *Prosopis juliflora* to Phytohormones at Different Germination Times

	Germination time		
	0 hrs	**48 hrs**	**96 hrs**
		Cytokinin	
Tamarindus indica	0.5647	0.9183	0.6338
Prosopis juliflora	1.0254	1.2695	1.0957
F-test	**		
SE±	0.0078		
$LSD_{0.05}$	0.0245		
$LSD_{0.01}$	0.0348		
		Gibberellin	
Tamarindus indica	0.4424	0.6150	0.7392
Prosopis juliflora	0.6285	0.6496	0.7747
F-test	*		
SE±	0.0270		
$LSD_{0.05}$	0.0851		
$LSD_{0.01}$	0.1210		
		Indole acetic acid	
Tamarindus indica	0.6019	0.5964	0.5174
Prosopis juliflora	0.2370	0.2289	0.2095
F-test	**		
SE±	0.0016		
$LSD_{0.05}$	0.0049		
$LSD_{0.01}$	0.0070		
		Abscisic acid	
Tamarindus indica	0.5561	0.5181	0.3997
Prosopis juliflora	0.1140	0.1141	0.1005
F-test	**		
SE±	0.0008		
$LSD_{0.05}$	0.0024		
$LSD_{0.01}$	0.0034		

* = $p<0.05$; ** = $p<0.01$; SE = standard error of the mean; LSD = lest significant difference

The higher germination in *P. juliflora* could be attributed to the higher cytokinin and gibberellin obtained in this study. These two hormones, cytokinin and gibberellin also occurred in relatively higher concentrations than the other two, indole acetic and abscisic acid. This generally suggests

that hormonal concentration has a greater role to play in germination, and cytokinin and gibberellin are the most important, judging by the recorded higher germination in *P. juliflora*. Conversely, the higher indole acetic acid and abscisic acid could explain the low germination experienced in *T. indica*. Literature has adduced the inhibitory action of abscisic acid to the inhibition of the cell cycle by activation of a residual G1 kinase and specifically delays enzymatic synthesis that is critical to seed germination [14], [34] – [35] Furthermore, these differences in phytohormone concentrations between *T. indica* and *P. juliflora* could be attributed to differences in imposed dormancy during maturation, seed coat thickness, embryo size and inherent hormonal levels and epigenetic regulation genes in the two species which according to Graber et al. [16] might also constitute differences. Dormancy is acquired during the maturation of seed including the formation of organs and nutrient storage as well as changes in the embryo size and weight followed by the acquisition of desiccation tolerance and dormancy [8], [29], [36]. Consequently, seed maturation results in inhibition of the cell cycle, decreased seed moisture, increased ABA levels, production of storage reservoirs and established dormancy [35].

The pattern and rate of germination in *Tamarindus indica* and Prosopis juliflora over fifteen days period was observed. The results showed that percentage germination as from 2 - 15 DAP, ranged from 4.0 - 61.4% and 4.1 - 68.1% in *T. indica* and *P. juliflora*, respectively. Thus, results from 3 - 15 DAP consistently depicted significantly ($p<0.05$) higher germination in *P. juliflora* than *T. indica*. Germination pattern over the fifteen days assessment period assumed logarithmic (sigmoid) pattern in both species, with distinct slow, rapid and then stable germination progress phases. The coefficient of determination revealed faster germination in *P. Juliflora* ($r^2 = 0.9403$) than *T. indica* ($r^2 = 0.9196$). Consequently, the regression coefficient (b-value) that defined the rate of germination in the two tree species, also indicated higher germination rate in *P. Juliflora* [$b = 28.256\ln(x)$] than *T. indica* [$b = 25.791\ln(x)$] (Figure 1).

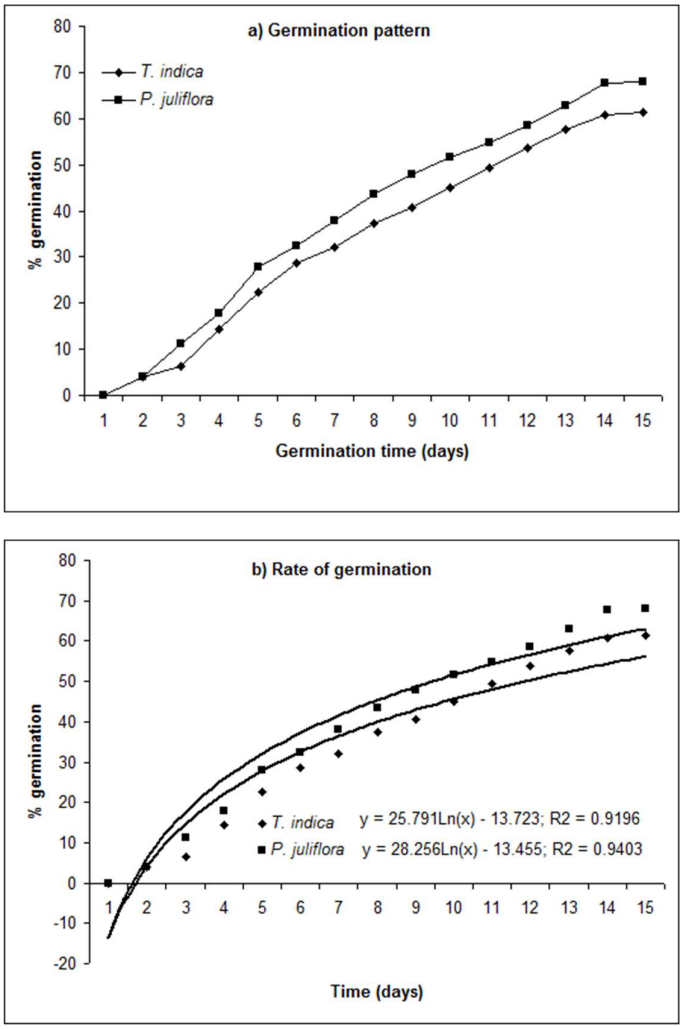

Figure 1. Comparison of a) Pattern and b) Rate of Germination in *T. indica* and *P. juliflora* Over Fifteen Days

The hormonal change in the two tree species over 0, 48 and 96 hrs. In general, the coefficient of determination (r^2) expressed highly significant ($p<0.01$) effects of the imbibition period on the concentration of hormones in both species, with distinct patterns among the four studied hormones. Fig. 2a expressed that changes in concentration of cytokinin followed a polynomial pattern, with distinct increasing (0 - 48 hrs) and decreasing (48 - 96 hrs) phases in both species. Consequently, the result indicated higher effects (r^2) of imbibition time on the concentration of cytokinin *T. indica* (86.34%) than *P. juliflora* (77.99%). However, both rates of increase (1.2726 vs 0.8388 mg/ml) in cytokinin concentration from 0 to 48 hrs and decrease (0.3046 vs 0.2067 mg/ml) from 48 to 96 hrs were relatively higher in *P. juliflora* than *T. indica*, respectively. In contrast, there was a highly significant ($p<0.01$) direct (positive) linear correlation between gibberellin concentration and germination, depicting an increase in the concentration of the hormone with an increase in time of germination in both species (Fig. 2b). The r^2 revealed higher effects of germination time in *T. indica* (99.12%) than *P. juliflora* (85.57%); thus the rate of increase in gibberellin concentrations in *T. indica* (0.1484 mg/ml) was higher than in *P. juliflora* (0.0731 mg/ml), despite the higher initial (0 hr) gibberellin

concentration in the dormant seed of *P. juliflora* (0.5381 mg/ml) than *T. indica* (0.3021 mg/ml). The pattern of change in indole acetic acid also differed, in which germination time exerted highly significant ($p<0.01$) negative effects, depicting a decrease in the concentration of indole acetic acid as imbibition progressed (Fig. 1c). The r^2 revealed higher effects of imbibition time on indole acetic acid in *P. juliflora* (94.67%) than *T. indica* (79.86%); conversely, both the initial indole acetic acid concentrations in the dormant seed of 0.6564 mg/ml and 0.2526 mg/ml, and the rates of decrease in hormone concentration of 0.0422 mg/ml and 0.0138 mg/ml were relatively higher in *T. indica* than *P. juliflora*, respectively (Figure 2).

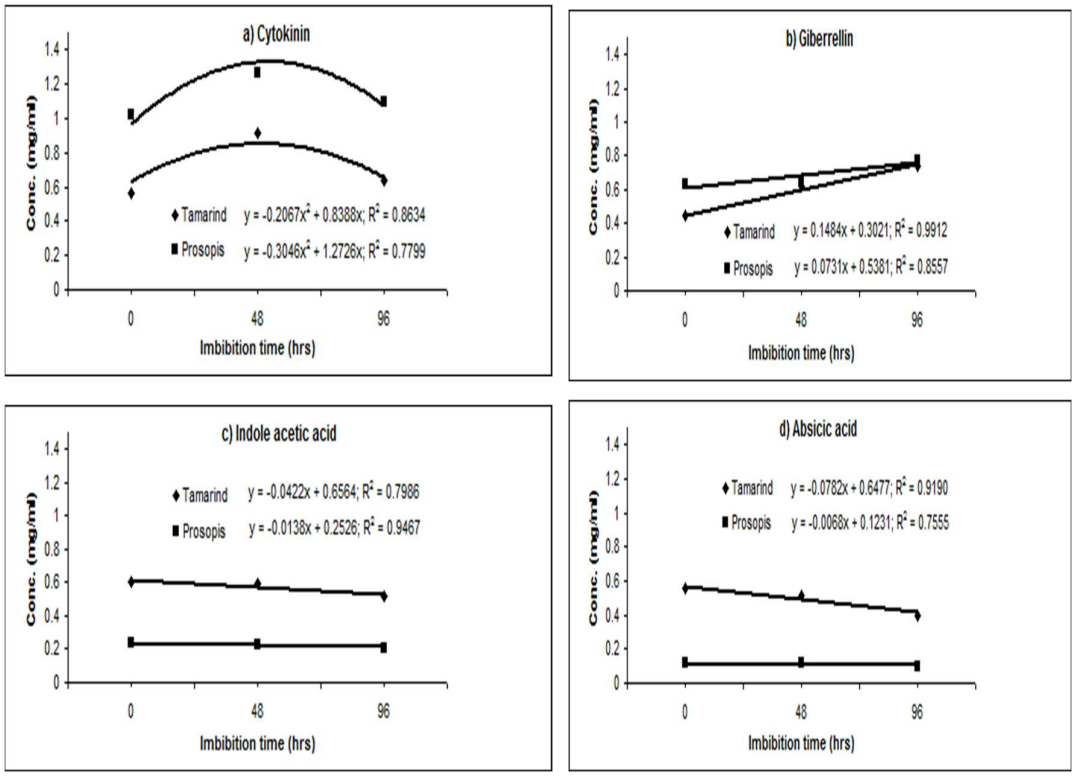

Figure 2. Pattern and Rate of Change in Hormonal Concentrations of a) Cytokinin b) Gibberellin c) Indole Acetic Acid, and d) Abscisic Acid in *Tamarindus indica* and *Prosopis juliflora*

4. Conclusion and Recommendation

Hormonal concentrations depended on imbibitions time. In conclusion, higher germination of *P. juliflora* is attributed to cytokinin and gibberellins, and the lower germination in *T. indica* to the higher inhibitory effects of IAA and ABA. Therefore, it is obvious that the importance of these hormones in the germination of these tree species cannot be overstated. The imbibe (water) imbibition triggerered hormonal responses by the imbibant; thus, imbibition is critical in eliciting the germination process in seed, through activation of hormonal activity that plays different roles during dormancy which varies in different plant species. However, the interaction effects of tree species x imbibition time indicated that hormone concentration in the dormant seeds, did not differ between plants species, however, after 48 and 96 hrs imbibition the

concentration of hormone in T. indica was higher than that of P. juliflora. However, the differences in imbibition in the two species could be attributed to seed size, seed coat thickness and oil contents that might have direct effects oIn conclusion, therefore, both imbibition and hormones played a critical role in the germination of *P. juliflora* than *T. indica*, despite the higher germination in *P. juliflora* compare with *T. indica*. It is recommended that further studies at different inbibition time with other plants hormones should be conducted with *T. indica* and *P. juliflora*. Molecular characterization should also be explored to evaluate the possible marker gene that is triggered by the time of inbibition in these tree species.

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