BIOTROPIA Vol. 29 No. 2, 2022: 124 - 133 DOI: 10.11598/btb.2022.29.2.1656

DEVELOPMENTAL MORPHO-ANATOMY AND GERMINATION OF THE SEEDS OF *Pterocarpus indicus* f. *echinatus* Willd. VARIANTS

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Received 8 September 2021/Accepted 10 December 2021

ABSTRACT

Previous studies on the embryo structure of legumes species had resulted in the division of the Fabaceae family into two great subfamilies based on embryo axis curvature. Research on seed morphology and anatomy adds to the knowledge of taxonomy, evolution and ecology. This study determined the seed developmental anatomy, pod and seed morphology as well as germination characteristics of the observed variants (T1 - small prickles; T2 - medium prickles; T3 - long prickles) of *Pterocarpus indicus* Willd. f. *echinatus* locally known as prickly narra in the Mount Makiling Forest Reserve (MMFR). Based on the anatomy of the root (radicle) and shoot apex, the formation of the leaf primordium in T2 seeds after radicle protrusion was more progressive. It was observed that the germination rate and the percentage were the highest in T2, where the apical dome was well-developed. The germination, pod and seed morphological characters as well as seed anatomical characters were proven to be systematically informative by showing significant differences among the variants.

Keywords: Fabaceae, Faboidae, meristem, pod, radicle

INTRODUCTION

The Fabaceae family consisting of 686 genera with more than 18,000 species is the third largest flowering plant family after Asteraceae and Orchidaceae (Mabberley 1997). The study of De Candolle (1825), resulted in the division of Fabaceae into two great subfamilies based on embryo axis curvature (i.e., Curvembriae and Rectembriae). The first subfamily contains the Faboideae, while the second one contains the Caesalpinioideae and Mimosoideae. Even though embryo axis curvature is currently not considered as the best character for primary divisions in the family, it may be one of the seed characters (especially hilar characters) used to divide the Faboideae from the other subfamilies (Oliveira & Paiva 2005). Faboideae is the largest of the Fabaceae subfamilies, with about 440 genera and 12,000 species (Polhill 1981). Certain seed characteristics are very helpful for faboid generic identifications i.e., aril, endosperm, radicle concealment by the cotyledons, cotyledon lobes over the radicle, overall radicle shape, radicle tip shape, and radicle length relative to that of the cotyledons (Kirkbride *et al.* 2003).

Capitaine (1912) stated the importance of legume seed morphology in legume classification and identification at the tribal, generic and specific levels. In the last 30 years, there has been a revival of interest in seed morphology and about 225 publications on the subject have been documented (Kirkbride *et al.* 2003).

Research on seed morphology and anatomy adds to the knowledge of taxonomy, evolution, and ecology of Angiospermae species (Cortez & Carmello-Guerreiro 2008). However, it is essential to emphasize that seed morphology generally presents little phenotypic plasticity. Conversely, embryological characters, typically

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constant in the genera, serve as an important indicator of taxonomic affinity (Von Teichman & Van Wyk 1991). According to Oliveira and Paiva (2005), there are only few descriptive and ontogenetic studies on seed structure causing difficulties in building the hypothesis of evolutive trends affecting seeds. Knowledge on fabaceous seeds is mainly related to hard seeds (Baskin & Baskin 1998; Baskin *et al.* 2000) while studies on seeds are limited, with the majority of such studies concentrating on species of agricultural interest, mainly soybean and bean (Qutob *et al.* 2008). Basic knowledge about seed characterization has proven its importance in dealing with problems in the field of seed technology and is essential in activities designed to maintain biodiversity and germplasm conservation. External morphology characterization of seeds has been used by various authors in species identification (Youngberg *et al.* 1998; Cappers & Bekker 2013). Characters mostly used include shape, weight, diameter, color and texture.

Populations of *Pterocarpus indicus* or narra over the natural range of distribution has declined over the years due to unselective cutting and overall habitat loss. Therefore, this species has been categorized under endangered category (IUCN v. 2021-1) while being categorized as critically endangered under the Philippine National Red List for Plants (DENR-DAO 2017-11). To conserve the remaining population, intensive research on the species have been conducted (Gazal *et al.* 2004; Krishnapillay *et al.* 1994; Xu *et al.* 2016). There are two recognized forms of narra (*P. indicus*) namely smooth narra (*Pterocarpus indicus* forma *indicus*) and prickly narra (*P. indicus* f. *echinatus*). However, some authors have recognized the presence of intermediate forms (Jøker 2000; Duke 1983). This may be attributed to the high degree of cross-pollination in *P. indicus*. There have been a few studies on morphology and field germination of the species, but there remains a dearth of information on the anatomy of the seeds, especially of *P. indicus* f. *echinatus*.

This study determined the developmental anatomy, pod and seed morphology as well as germination characteristics of the observed variants of *P. indicus* f. *echinatus* collected from MMFR to assess the extent of variation in this form.

MATERIALS AND METHODS

Study Site and Seed Collection

Seeds were collected in the Mt. Makiling Forest Reserve (MMFR) (14°8' N and 121°12' E) in 2018. Flowering of *P. indicus* f. *echinatus* starts in January and peaks around April and May. Regular fruiting season starts from January to July or September to November. Selection of mother trees was based on characters that distinguish *P. indicus* f. *echinatus* trees from the smooth form; leaflets distinctly more obovate with acuminate apex and inner bark relatively whitish to yellowish. However, the observed variation in prickles length was confirmed during pod maturity, usually four months after fruit bud formation. Pods were collected and air-dried. Seeds were extracted from the pod using scissors and forceps. Pods are disc-shaped, flat and have winged margins or samara. Unlike most legumes, the *Pterocarpus* pod is indehiscent and is wind dispersed. The pod also floats in water and can be water dispersed. Around 5 cm across, the pod has a central woody corky bulge containing 1 - 3 seeds. The seeds have very thin seed coats (Orwa *et al.* 2009).

Morphological Measurement

Pod and seed characters were described and compared among the three variants. For the pod morphology, different variants of *P. indicus* f. *echinatus* were categorized not only by the length of the prickles but also by the shape and size of the pods, number of seeds per pod, and number of prickles. For the seed morphology, seed length and width were determined using a ruler (mm). The mean for every characteristic was calculated.

Anatomical Measurement

Permanent sections of the germinated seeds were obtained using a modified histological paraffin technique by Johansen (1940). The seeds were fixed using Formalin: Acetic Acid: Alcohol (FAA) solution for two weeks, dehydrated using different percentages of alcohol and infiltrated with paraffin wax (Tables 1 and 2). The seeds were then embedded in paraffin wax and mounted on wooden blocks measuring 2 x 2 x 2 cm. The samples were sectioned using a rotary microtome with a thickness of 10 to 15 μm. The resulting paraffin ribbons were then mounted on glass slides, decerated, stained with Safranin and counterstained with Fast Green. Drops of Entellan were added over the stained sections prior to the addition of cover slips and were then air-dried.

Photomicrographs of the seed's embryo were obtained using Optika microscope under 400x magnification. The thickness of different tissues (root apical meristem, shoot apical meristem, procambium, ground meristem, protoderm and leaf primordium) were measured using the Optika software. The development of the embryo was observed starting from the ungerminated seeds until the radicle protrusion and elongation of the embryo. Haupt (1953), Fahn (1967), Bell (2008) and Shipunov (2020) were followed to describe the developmental anatomy of the seed.

Table 1 Paraffin schedule (fixation, dehydration, infiltration and embedding)

Solution	Procedure per day	Duration in solution (hours)
Fixation (FAA-A and FAA-B mixture)		One week
50% EtOH	Day 1 1st dehydration	1 hour
50% EtOH	Day 1 2nd dehydration	1 hour
50% EtOH	Day 1 3rd dehydration	1 hour
50% EtOH	Day 1 4th dehydration	1 hour
J1	Day 1	2 hours
J2	Day 1	Overnight
J3	Day 2	2 hours
J4	Day 2	2 hours
J5	Day 2	2 hours
J6	Day 2 (in warm place; vial uncorked)	Overnight
J6	Day 3 (3 TBA changes every 2 hours)	6 hours
TBA (1)	Day 3 (uncorked at room temperature) (fumehood)	1 - 4 hours
TBA + paraffin pellets	Day 3 uncorked at 65 °C)	3 to 4 hours

Table 2 Staining schedule for *Pterocarpus indicus* samples

Solution	Time (minutes)
Xylene	15
TBA	15
Absolute Ethanol	15
95% Ethanol	15
50% Ethanol	15
H_2O	3
Safranin	30
H_2O	3 to 4 rinses
50% Ethanol	15
95% Ethanol	15
Fast Green	5
95% Ethanol	15
Absolute Ethanol	15
TBA	30
Xylene	30

Germination

Viability test was conducted to *P. indicus* f. *echinatus* seeds via flotation method (Dayan & Reaviles 1995). Viable seeds were washed with running water and were arranged in sterilized petri dishes containing filter paper with water. The set-up was done in the Microtechnique Laboratory of the Department of Forest Biological Sciences, College of Forestry and Natural Resources, University of the Philippines Los Baños (DFBS, CFNR, UPLB). The seeds are considered germinated when visible protrusions of plumule is observed. For the germination percentage (Equation 1) and germination rate (Equation 2) (Awasthi *et al.* 2016), the following formulae are used:

$$\text{Germination Percentage (\%)} = \frac{\text{Number of Total Germinated Seeds}}{\text{Total Number of Seeds Tested}} \times 100 \quad (1)$$

$$\text{Germination Rate} = \frac{\text{Number of Germinated Seeds}}{\text{Day of First Count}} + \text{- -} + \frac{\text{Number of Germinated Seeds}}{\text{Day of Final Count}} \quad (2)$$

Experimental Design and Analysis

The germination experiments used a simple Complete Randomized Design (CRD) with three treatments having four replicates of 50 seeds each. The experiment included three variants (as treatments) of *P. indicus* f. *echinatus,* namely: T1 = small prickles; T2 = medium prickles; and T3 = long prickles.

The One-way Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were used to test for significance of the mean differences in terms of pod and seed morphological characters (pod diameter, length of prickles, number of prickles, seed length and seed width) and germination characteristics of *P. indicus* f. *echinatus.* Analyses were performed using R Studio version 4.1 (R Studio Team 2020).

RESULTS AND DISCUSSION

Morphological Features

Certain characters (pod diameter, length of prickles and number of prickles) displayed variation among variants and were found to be potentially informative, whereas other characters (pod shape, presence of prickles and seed shape) were observed to be similar in all variants studied.

All *P. indicus* f. *echinatus* pods found in this study were thin, papery-winged and disc-shaped. Generally, the pods had bulge at the center containing the seeds. Pod usually has a diameter of 5 - 8 cm, but all have their own unique characteristics. Pods of T1 variant contained up to four (4) seeds, while only 1 - 3 seeds were found in T2 and T3 variants. On the other hand, the pod diameter had nearly significant variations among the three variants (P = 0.0535), while both the length of prickles (P = 0.000) and the average number of prickles (P = 0.000) were significantly differentiated among the variants. T2 had numerous prickles (95.095±0.461) per pod compared to those of T1 and T3. Lastly, T3 had soft and the longest prickles (8.764±0.027) while T2 and T1 had hard prickles and shorter prickles (Table 3).

Table 3 Pod morphological characteristics of the *Pterocarpus indicus* f. *echinatus* variants collected from MMFR

Morphological characteristic	Variant		
	T1	T2	T3
Pod shape	Disc-shaped	Disc-shaped	Disc-shaped
Pod diameter (cm)	6.450 ± 0.136^{b}	6.746 ± 0.071^{a}	6.524 ± 0.047^{ab}
No. of seeds	1 - 4	1 - 3	1 - 3
Length of prickles (mm)	6.248 ± 0.026^{c}	7.230 ± 0.028^{b}	8.764 ± 0.027^{a}
Number of prickles	62.095 ± 0.809^{b}	95.095 ± 0.461^{a}	55.524 ± 0.562^{c}
Stiffness of prickles	Hard	Hard	Soft

Notes: T1 = small prickles; T2 = medium prickles; T3 = long prickles.

Table 4 Seed morphological characteristics of the *Pterocarpus indicus* f. *echinatus* variants collected from MMFR

Morphological character	Variant		
	T1	T2	T3
Seed shape	Falcate	Falcate	Falcate
Seed color	Orange-brown	Orange-brown to reddish-brown	Reddish-brown to brown
Seed width (mm)	5.167±0. 122^{ns}	4.905±0. 127^{ns}	5.095±0. 159^{ns}
Seed length (mm)	13.571±0.316^{ns}	13.333±0.349^{ns}	12.905±0.325^{ns}

Note: ^{ns} = no significant difference among the variants at $P < 0.05$.

Seed color of variants varied from orange brown to reddish-brown. Seed width (P = 0.380) and seed length (P = 0.354) showed no significant variations among the three variants (Table 4). In this study, the seeds of *P. indicus* f. *echinatus* were flat, falcate-shaped and had almost similar width and length ranging from 4.905 to 5.167 mm and 12.905 to 13.571 mm, respectively.

The pod description of *P. indicus* f. *echinatus* in this study is consistent with the reports of Orwa *et al.* (2009), Thomson (2006), Francis (2002) and Flores *et al.* (2021) having indehiscent disc-shaped and flat pod with winged margins. About 5 cm across, it has a central woody-corky bulge containing several seeds. Dayan and Reaviles (1995) reported *P. indicus* f. *echinatus* pod length of 5 - 6 cm including the wing (1.5 - 2.5 cm), while Rojo (1977) and Duke (1983) reported 4 - 7 cm and 4 - 6 cm pod diameter, respectively. In this study, 5 - 8 cm pod diameter was observed. Flores *et al.* (2021) confirmed that *P. indicus* f. *echinatus* has pod size ranging from 5 - 8 cm. Light environment and soil moisture influence fruit quality including fruit size and color (Kozlowski & Pallardy 1997; Raina 2003).

Different literatures reported various number of seeds per pod, such as Francis (2002) reported 1 - 4 seeds per pod, Orwa *et al.* (2009) reported 1 - 3 seeds per pod, while Jøker (2000) and Duke (1983) reported 1 - 2 seeds per pod. In this study, the T2 and T3 variants of *P. indicus* f. *echinatus* contained 1 - 3 seeds per pod while T1 has 1 - 4 seeds per pod. According to Kelly (1984), the number of seeds per seeding plant is one of the basic parameters necessary for a description of the population dynamics of species which does not reproduce vegetatively. Moreover, it was emphasized that in situations where the number of seeds per fruit was found to vary within years or among treatments, there seemed to be a stable relationship between fruits per plant and seed numbers.

In terms of prickles, T2 was found to have numerous hard prickles, while T1 and T3 were found to have a lesser number of prickles (Table 3), which is in agreement with the study of Flores *et al.* (2021). Kellogg *et al.* (2011) defined prickles as outgrowths of epidermal tissues and can provide a simple developmental system for the study of the control of cell proliferation and growth. Prickles constitute one of the many types of plant defense against vertebrate herbivore (Janzen & Martin 1982; Cooper & Owen-Smith 1986; Milewski *et al.* 1991 as cited by Ronel & Lev-Yadun 2012).

For seed morphology, seed width and seed length in this study were not significantly different among *P. indicus* f. *echinatus* variants. Jøker (2000) and Thomson (2006) reported 6 - 8 mm seed length for *P. indicus* with brown papery testa. According to Harper (1977) and Silvertown (1989) as cited by Chacon *et al.* (1998), seed size is a life history trait that may affect the fitness of the parent's plants and the population regeneration process. Large seeds tend to have a positive effect on germination. In this study, T2 had a significantly similar germination percentage to that of T1, but different from that of T3 (Table 5). The morphology distinction of angiosperm seeds and the relative consistency of seed structures in narrow taxonomic units allow the use of seed characteristics in taxonomic research (Esau 1977). The most significant seed morphological characters are shape, size, testa surface, the position of hilum and the presence or absence of specialized structures such as aril, caruncle or elaiosomes. The differences in bristle-like prickles or spicules in terms of length and number as well as number of seeds per pod as observed in *P. indicus* f. *echinatus* may support the argument of Jøker (2000) and Duke (1983) that intermediate forms may occur.

Anatomical Features

In the root apex, the root cap had become more developed after protrusion, consisting of 7 - 10 layers of cells. For the germinated seeds, the root tip of T2 was more round compared to the root tips of T1 and T3 (Fig. 1).

The procambium became more evident after radicle protrusion and was the thickest in T3. The ground meristem also increased in thickness after radicle protrusion. The Shoot Apical Meristem (SAM) of the embryo was dome-shaped which was observed in the embryo of all *P. indicus* f. *echinatus* variants. Well-developed leaf primordium was observed in all of the embryos after radicle protrusion. On the other hand, the formation of leaf primordium in T2 seeds after radicle protrusion was more progressive (Fig. 2).

SAM is essentially a dome-shaped structure with undifferentiated cells at the tip, surrounded by a differentiating peripheral zone that participates in leaf formation. A well-developed apical dome is directly related to high germination frequency (Corredoira *et al.* 2002). A less developed apical dome would mean lesser germination frequency. In this study, well-developed leaf primordium can be observed in all of the embryos after radicle protrusion (Fig. 2).

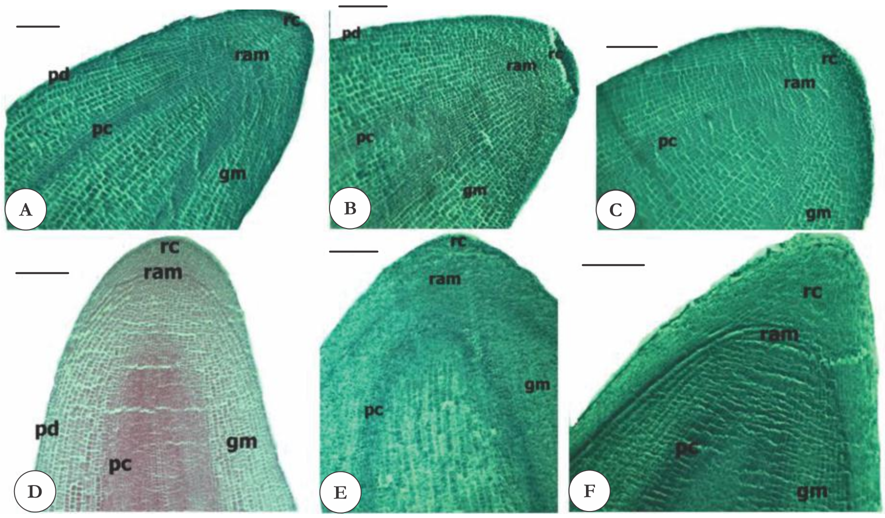

Figure 1 Longitudinal section of the root apex of the variants of *Pterocarpus indicus* Willd. f. *echinatus* collected from MMFR

Notes: A-C = Before protrusion; D-F = After protrusion.
A and D = T1 root apex; B and E = T2 root apex; C and F = T3 root apex.
rc = root cap; *ram* = root apical meristem; *pc* = procambium; *pd* = protoderm; *gm* = ground meristem.
Scale bar = 200 µm.

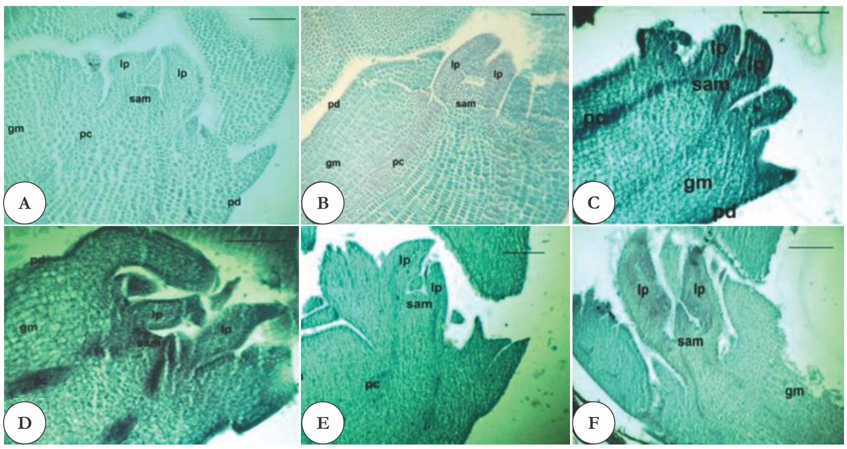

Figure 2 Longitudinal section of the shoot apex of the variants of *Pterocarpus indicus* Willd. f. *echinatus* collected from MMFR

Notes: A-C = Before protrusion; D-F = After protrusion.
A and D = T1 root apex; B and E = T2 root apex; C and F = T3 root apex.
sam = shoot apical meristem; *pc* = procambium; *pd* = protoderm; *lp* = leaf promordium.
Scale bar = 200 µm.

Seed anatomical characters showed value in verifying taxonomic relationships (Esau 1977). *P. indicus* f. *echinatus* was found to have a root tip cap of 7 - 10 layers of cells. In this study, the root tip cap of T2 variant was found to be round, while those of T1 and T3 were pointed. Roue *et al.* (2020) proved in their study that root tip cap structure affects apex penetration and reorientation, in which rectangular-shaped root tip cap showed enhanced penetration abilities compared to the pointed root tip cap.

Kumpf and Nowack (2015) mentioned that modern plant biology has unraveled that many of the functions that Darwin attributed to the root tip are actually accomplished by the root tip cap, which is a multi-layered dome of spindle-shaped parenchyma cells that overlies the growing root tip (Iijima *et al.* 2008). The root tip cap surrounds and protects the meristematic stem cells at the growing root tip. In addition, the root tip cap shows a rapid turnover of short-lived cells regulated by an intricate balance of cell generation, differentiation and degeneration. In *Arabidopsis thaliana*, the root tip cap cells are actively killed and degraded on the root surface, while a limited amount of short-lived 'border-like' cells are released into the rhizosphere (Durand *et al.* 2009; Fendrych *et al.* 2014).

Based on the classification of Kumpf and Nowack (2015), *P. indicus* f. *echinatus* can be considered to have a closed meristem structures which form their cell lineages from specific stem cells, including the root tip cap lineage, which shows a defined root cap stem cells and a layered root cap structure. While new root cap cells are constantly produced by root cap stem cells in an indeterminate fashion, the size and cell number of the root cap are determinate (Barlow 2003).

The procambium in this study became more evident after radicle protrusion and was the thickest in T3. The ground meristem also increased in thickness after radicle protrusion. The procambium provides the basis for the differential modulation of long-distance transport capacities and plant body stability (Jouannet *et al.* 2015).

Germination

Results of germination percentage and germination rate showed significant differences for the three variants. Germination percentage and germination rate were both the highest in T2 with 82.67% and 42.86, respectively (Table 5; Fig. 3).

Table 5 Mean germination percentage and rate ± SE of the *Pterocarpus indicus* f. *echinatus* variants collected from MMFR

Parameter	Variant		
	T1	T2	T3
Germination percentage (%)	60±1.15^{ab}	82.67±3.53^{a}	50.667±0.667^{b}
Germination rate (No. of seeds per nth day)	16.73±1.15^{b}	42.86±1.95^{a}	23.46±1.79^{b}

Note: numbers followed by the same letter are not significantly different based on Duncan test at $P < 0.05$.

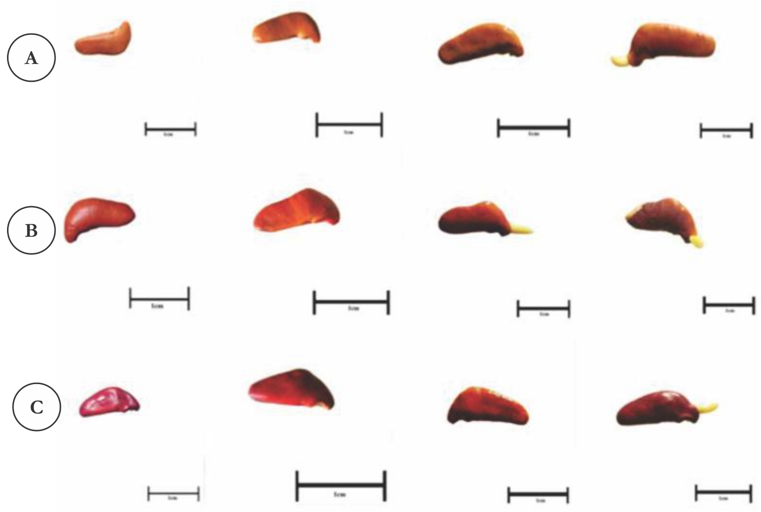

Figure 3 Seeds showing germination up to radicle protrusion
Notes: A = T1; B = T2; C = T3. Day 0 to Day 3 from left to right. Scale bar = 1 cm.

In this study, seeds began to germinate 3 - 4 days after sowing, which is similar to the observation of Thomson (2006). Germination usually commences with the uptake of water by the dry seed through imbibition and is completed when the radicle extends to penetrate the structures that surround it (Bewley 1997). In the study of De Sedas *et al.* (2019) the osmotic effect due to salinity was the main inhibitory factor that reduced germination of inland neotropical tree species such as *Minquartia guainensis, Apeiba menbranacea, Ormosia ccoccinea* and *Ochroma pyramidale*.

High germination percentage and germination rate in T2 (Table 5; Fig. 3) can be attributed to various factors. In the study of Vozzo (2003) as cited by Luna *et al.* (2014), tropical species that benefit from reagent, such as hydrogen peroxide, included *Albizia* species and camphor tree seeds. Valio and Scarpa (2001) proved that the seeds germination percentage and rate of seven tropical pioneer species in Brazil (*Cecropia hololeuca, C. pachystachya, C. glazioui, Solanum gracillimum, S. granuloso-leprosum, S. tabacifolium* and *Miconia chamissois*) were significantly higher in the irradiated condition than in shaded condition. On the other hand, seed germination rate of *Peltophorum dubium* varied with water potential treatment (Daibes & Cardoso 2020).

CONCLUSION

The germination, pod and seed morphological characters and seed anatomical characters proved to be informative by distinguishing significant differences among the observed variants of *P. indicus* f. *echinatus*. The variants can be differentiated by the average number of prickles, length of prickles, stiffness of prickles, pod diameter, number of seeds per pod and seed color. The formation of the leaf primordium in T2 seeds after radicle protrusion was more progressive. The observed variants did not differ in their germination characteristics. Differences of shoot apical meristem can be observed based on the development of the apical dome of each variant, which is related to the germination percentage of each variant. This study on *P. indicus* f. *echinatus* is limited to MMFR only and examines seed only and does not include other parts of the tree. Also, this study does not include parameters such as seed coat and fruit anatomy. It is therefore recommended that further research, such as molecular and DNA analyses, be conducted in the future to shed light on the observed variation in this study. Characters such as seed coat anatomy can add more knowledge on the separation of the variants being studied.

ACKNOWLEDGMENTS

The authors wish to thank the Department of Forest Biological Sciences and the Makiling Center for Mountain Ecosystems (MCME), CFNR-UPLB for allowing the use of their facilities to conduct this study. This study is partially supported by the DOST-PCAARRD-funded project "Germplasm Conservation of Selected Indigenous Forest Trees in MMFR".

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