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Morphology, Storage Behavior, Dormancy, Germination Pattern and Chemical Composition of *Tabernaemontana pandacaqui* Lam. Seeds

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ABSTRACT

Background: Seed characteristics of *Tabernaemontana pandacaqui* Lam., an indigenous medicinal plant species from the Philippines, were investigated to help identify appropriate conservation strategies.

Methods: Mature seeds were tested for viability at different dormancy-breaking treatments and storage conditions. Seeds were also characterized morphologically and the seedling developmental pattern was established using the Biologische Bundesanstalt, Bundessortenamt and CHemische Industrie (BBCH) scale. We also determined the preliminary chemical composition through qualitative phytochemical screening and proximate analysis.

Result: Seeds of *T. pandacaqui* are obovate in shape with a 1000-seed weight of 20.99±0.62 g at 4.7% moisture content. Removal of the aril from the fresh seeds improved germination from 30%±0.98 to 84%±0.71 and shortened the time to seedling emergence from 45 days to 4 days after sowing. The seeds can tolerate drying to 4.7% moisture content and storage at -10°C for 3 months; thus, they have orthodox storage behavior. The germination pattern is epigeal phanerocotylar type, wherein the foliaceous cotyledons are raised (BBCH 09) and unfolded above the soil surface (BBCH 10). The seedling then develops an ovate to elliptic-shaped first true pair of leaves (BBCH 12) completing the germination process. The pre-dried seeds also have a high oil content of 28.7407±0.09316% with a strong presence of alkaloids and saponins. This indicates that the seed is amenable to ex-situ conservation through seedbanking, but its viability must be monitored in shorter intervals due to its high oil content.

Key words: BBCH scale, Dessiccation tolerance, Phytochemical content, Proximate composition, Seedling development.

INTRODUCTION

Tabernaemontana pandacaqui Lam. (Apocynaceae) is an evergreen shrub or a small tree with ornamental flowers and fruits. It is native to South China up to the Pacific (Royal Botanic Gardens Kew, 2019). In the Philippines, it is commonly found in thickets at low elevations and is present from Babuyan islands to Mindanao (Merril, 1926). *T. pandacaqui* is widely studied for its phytochemical and pharmacological components; it has anti-inflammatory, antipyretic, analgesic and antibacterial activities which are attributed to tabernaemontanine and akuammicine alkaloids found in the stem and leaves, respectively (Blasco *et al*., 2014; Taesotikul *et al*., 2003; Abe *et al*., 1993; Boonchuay *et al*., 1976). In the Philippines and Thailand, the sap and leaves of *T. pandacaqui* are used as an emmenagogue and emollient to wounds, respectively (Chua and Horsten, 2001).

T. pandacaqui is mainly propagated through seeds. However, information on seed morphology and germination pattern remains fragmentary. In addition, no record has been found on the seed storage behavior and chemical composition of *T. pandacaqui* (Chua and Horsten, 2001; Royal Botanic Gardens Kew, 2019). Data on its seed storage behavior and chemical composition are fundamental in developing appropriate conservation and management strategies (Hong *et al*., 1996). Furthermore, detailed information on its germination pattern and dormancy-breaking technique is important in establishing germination techniques and nursery management. Given the medicinal

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potential of *T. pandacaqui*, the study aimed to characterize the seed morphological features, evaluate the storage behavior, establish the initial dormancy-breaking technique, elucidate the detailed germination pattern and determine its preliminary chemical composition for genetic resource conservation and management.

MATERIALS AND METHODS

Plant materials

Mature seeds of *T. pandacaqui* (GB 67966) collected from plants maintained at the Crop Breeding and Genetic Resources (CBGR) Laboratory, Institute of Crop Science (ICropS), College of Agriculture and Food Science (CAFS), University of the Philippines Los Baños (UPLB) were used. Clean, undamaged and undeformed fruits and seeds were utilized for characterization. Seeds were sterilized by soaking in 5% sodium hypochlorite for five minutes and subsequently rinsed with distilled water prior to subjecting to dormancy-breaking treatments and seed storage behavior experiment.

Fruit and seed characterization

Morphological features of ten randomly selected mature fruits were observed. The number of seeds per fruit was recorded. Ten randomly selected seeds were used to measure and observe the shape, color and size (length, width, thickness). The weight of eight 100-seed replicates was used to determine the 1000-seed weight. The 1000-seed weight was computed using the formula:

$$\text{Weight of 1000 seeds} = \frac{\Sigma \text{ Weight of 100 seed replicates}}{\text{Number of 100 seed replicates}} \times 100$$

Seed size and 1000-seed weight were calculated as the mean±standard error of the mean. The basis for characterizing the color was the Royal Horticultural Society (RHS) Color Chart Sixth Edition (2015).

Seed dormancy

To determine the dormancy behavior and germination percentage, clean and sterilized seeds were subjected to six treatments at room temperature: (1) control, seeds with aril; (2) removal of arils (3) scarification using sandpaper; (4) presoaking in 0.2M KNO_3 for 12 hours; (5) presoaking in 100 ppm GA_3 for 12 hours; (6) presoaking in distilled H_2O for 12 hours. For each treatment, two replicates of 100 *T. pandacaqui* seeds were sown in sterilized sand. Seeds with approximately 2 mm radicle emergence were considered germinated.

Seed storage behavior

A modified method to determine seed storage behavior, based on Hong and Ellis (1996), was used. Three replicates of 100 fresh seeds were tested for germination after the following storage conditions: (1) fresh seeds at 14.35% moisture content (MC) in ambient temperature, (2) slow-dried seeds at 4.7% MC and stored in -10°C for 3 months. Seed moisture content was determined using the modified International Seed Testing Association (ISTA) low constant temperature oven method.

Seedling development

Following the protocol of Gentallan *et al*. (2018), the detailed germination pattern of *T. pandacaqui* was elucidated based on the extended Biologische Bundesanstalt, Bundessortenamt and CHemische Industrie (BBCH) scale by Hack *et al*. (1992). Thirty *T. pandacaqui* seeds from the standard germination set-up were identified and observed for growth and development. The BBCH scale of the growth stages is represented by a two-digit code using numbers from 0 to 9 in ascending order. The first digit signifies the principal growth stage while the second digit code is the secondary growth stage.

Preliminary phytochemical analysis

Sample preparation

Seeds of *T. pandacaqui* were pre-dried at 50°C for 24 hours, manually ground and pulverized using mortar and pestle and passed through an 18-mesh sieve. The powdered sample was stored in an air-tight container until further use.

Sample extraction

The extraction process was carried out following the method described by Doctor and Manuel (2014) with modifications. Approximately 10 grams of the powdered seeds were macerated in methanol at room temperature using a laboratory blender and then suction filtered to collect the seed extract. This was repeated twice. The crude extract was then concentrated at 50°C and stored under refrigerated conditions until further use.

Phytochemical analysis

Qualitative phytochemical analysis of the crude extract was determined following the method with modifications.

Alkaloids

Approximately 0.5 g of seed extract was diluted in distilled water and mixed with 5 mL of 0.1 N HCl solution in a hot water bath and filtered while hot. The filtrate was then added with 2-3 drops of Wagner's reagent. The formation of a reddish-brown precipitate indicates the presence of alkaloids.

Flavonoids

The crude extract was diluted in 0.1 N NaOH solution using a 1:1 (v/v) extract-NaOH solution ratio. The development of yellow color indicates the presence of flavonoids.

Saponins

Ten milliliters of distilled water was added to 0.5 mL seed extracts in a test tube and shaken vigorously. The formation of stable froth that persists for 5-10 minutes indicates the presence of saponins.

Tannins

Two to three drops of the crude extract were diluted in 1 mL of distilled water and the resulting solution was clarified through filtration. The filtrate was then added with a few drops of 1% ferric chloride solution. Development of bluish to black color indicates the presence of tannins.

Proximate analysis

The proximate composition (moisture content, ash, crude fiber, crude fat, crude protein and total carbohydrates) of pre-dried powdered seed samples was determined using the standard methods described by AOAC (2000).

Statistical analysis

Analysis of variance (ANOVA) and pairwise mean comparison using the least significant difference (LSD) test was used to determine statistically significant differences between means of the dormancy-breaking treatments at α=0.05. Germination (%) data were transformed to the arcsin of the square root of the fraction value before subjecting them to ANOVA.

RESULTS AND DISCUSSION

Fruit and seed characteristics

Tabernaemontana pandacaqui bears smooth to hairy, orange to red-orange capsules at maturity. The fruit is composed of two lateral capsules containing an average of 56 brown-colored seeds that are fully enclosed by a vivid reddish-orange aril. Seeds are obovate in shape with respect to the point of radicle emergence. The surface is wrinkled with longitudinal grooves and the aril is attached to the deep hilar groove. Seeds are relatively small being 6.92±0.19 mm in length, 4.69±0.18 mm in width, 3.16±0.17 mm in thickness with a 1000-seed weight of 20.99±0.62 g at 4.7% moisture content. It has ruminated endosperm corresponding to the description of Endress and Bruyns (2000) of *Tabernaemontana* species.

Seed dormancy

De-arillated *T. pandacaqui* seeds started germinating 4 days after sowing (DAS) while arillated seeds were observed to have induced dormancy with non-uniform germination starting at 45 DAS until 90 DAS. Germination of arillated seeds (30%) was significantly lower compared to de-arillated seeds with 84% germination. Scarification of seeds using sandpaper improved the germination of *T. pandacaqui* from 84% to 94%. Presoaking treatments of de-arillated seeds in 100 ppm GA_3, distilled water and 0.2 M KNO_3 did not significantly increase the germination of *T. pandacaqui* with 91%, 89% and 88% germination, respectively (Table 1). This demonstrates that the arils of the seeds must be removed to ensure a higher germination rate during regeneration or propagation. Unlike in nature where the aril creates favorable moisture conditions such as in the case of *Colophospermum mopane* (Jordaan and Wessels, 1999), arils may contain inhibitory substances that delay germination (Lange, 1961; Rodriguez *et al*., 2019). Aside from this, arils may prevent water imbibition by acting as "plugs" (Rolston, 1978) and arillated seeds were also susceptible to pathogen infestation during viability testing. Aril removal or scarification increases seed germination rates such as in the case of *Iris* spp. (Blumenthal *et al*., 1986) and *Passiflora* spp. (Colombo *et al*., 2018).

Table 1: Seed germination and dormancy breaking treatments for *Tabernaemontana pandacaqui*.

Dormancy breaking treatments	Germination (%)
Arillated	30^{c}
De-arillation (D)	84^{b}
D+Scarification using sand paper	94^{a}
D+Presoaking in 0.2 M KNO_3 for 12 hours	88^{ab}
D+Presoaking in 100 ppm GA_3 for 12 hours	91^{ab}
D+Presoaking in distilled H_2O for 12 hours	89^{ab}

^{a,b,c} Means followed by a different superscript letter are significantly different at α=0.05 using least significant difference (HSD) Test.

Seed storage behavior

Freshly extracted mature de-arillated seeds have a moisture content of 14.35% and seed viability of 90%. Seeds that were slow-dried to 4.7% MC and stored at low temperature (-10°C) for three months have comparable seed viability of 87%. Since the seeds can tolerate drying up to 4.7% and freezing up to -10°C for three months, the seeds were considered to be desiccation-tolerant and were categorized as having orthodox storage behavior (Roberts, 1973). This is the first report on the seed storage behavior of *Tabernaemontana pandacaqui* Lam.; however, plants on the same genus, *Tabernaemontana coffeoides* Bojer ex A.DC., *Tabernaemontana elegans* Stapf and *Tabernaemontana pachysiphon* Stapf were documented to have orthodox seeds (Royal Botanic Gardens, Kew, 2019). The character of *T. pandacaqui* seeds concurs with the general characteristics of an orthodox seed, *i.e*. smaller size and lower initial moisture content than recalcitrant seeds (>50%) at physiological maturity (Copeland and McDonald, 2012). Orthodox-seeded species are long-lived (Roberts, 1973) and can be stored long-term at about -18°C and 5% moisture content (Ellis *et al*., 1985). Hence, *T. pandacaqui* can be conserved in seed genebanks. Moreover, further ultra-drying experiments could yield cost-efficient conservation strategies of *T. pandacaqui* seeds.

Seedling development

The germination pattern of *T. pandacaqui* seeds was explained by germination (stage 0) and leaf development growth stages (stage 1) of the BBCH scale (Table 2). Radicle emergence of de-arillated fresh *T. pandacaqui* seeds was observed as early as 4 DAS (BBCH 05). The radicle elongates and develops root hairs and lateral roots (BBCH 06). Hypocotyl breaks through the seed coat (BBCH 07), elongates forming the hypocotyl arch (BBCH 08) and

Table 2: Phenological growth stages and BBCH identification keys of *Tabernaemonatana pandacaqui.*

Code	Description
Principal growth stage 0: Germination	
00	Fresh seed
01	Beginning of seed imbibition
03	Seed imbibition complete
05	Radicle emergence
06	Radicle elongation; formation of root hairs
07	Hypocotyl with cotyledons breaking through the seed coat
08	Hypocotyl reaches the soil surface; hypocotyl arch is visible
09	Emergence: hypocotyl with cotyledons emerged above the soil surface
Principal growth stage 1: Leaf development	
10	Cotyledons completely unfolded
12	2 leaves (first pair) unfolded
14	4 leaves (second pair) unfolded



Fig 1: Seedling development of *Tabernaemontana pandacaqui* depicted according to the BBCH scale.

subsequently emerges above the soil surface with the cotyledons remaining inside the seed testa (BBCH 09) (Fig 1). Ovate foliaceous cotyledons emerged from the testa (BBCH 10) that were followed by the appearance of leaf primordia that developed into the first true pair of leaves (BBCH 12). The first true leaves are ovate to elliptic in shape and glabrous with smooth and entire margins. The seedling development pattern of *T. pandacaqui* exhibits the usual phanerocotylar epigeal type of germination with foliaceous cotyledons that have photosynthetic activity (Perez-Harguindeguy *et al*., 2016). This species exhibits the *Macaranga* type of seedling development wherein the paracotyledons are exposed and raised above the ground (De Vogel, 1979). Its characteristic non-plastic germination pattern indicates that positioning studies during planting can be undertaken to ensure seedling establishment.

Preliminary chemical composition

This is the first report of the proximate and phytochemical composition of *T. pandacaqui* seeds. The phytochemical screening showed the presence of alkaloids, flavonoids and saponins on the methanolic extracts of *Tabernaemontana pandacaqui* seeds (Table 3). The presence of these phytochemicals is consistent with the methanolic extracts of *Tabernaemontana dichotoma* Roxb. seeds (Mahadimane and Chandra, 2020). The presence of flavonoids, however, can increase seed longevity. Having antimicrobial properties, flavonoids, particularly when localized in the seed coat, may provide chemical protection against pathogen infection (Treutter, 2006). Similarly, alkaloids may also provide this effect (Dey *et al*., 2017; Gruyal and Medina, 2019). Flavonoids can also promote longevity in storage by scavenging reactive oxygen species inducing cellular level

Table 3: Preliminary phytochemical properties of T. *pandacaqui* seeds.

Phytochemical	Result
Alkaloids	++
Flavonoids	+
Saponins	++
Tannins	-

++strong presence of phytochemicals.
+weak presence of phytochemicals.
-absence of phytochemicals.

Table 4: Proximate composition of *T. pandacaqui* seeds*.

Parameter	Result (%)
Moisture content (dry basis)	4.4515±0.0011
Crude fat	28.7407±0.09316
Crude protein	13.8581±0.2331
Crude fiber	39.1949±1.5458
Ash	1.8979±0.01075
Nitrogen free extract (NFE)	11.7475±1.9050
Total carbohydrates	

*Note: Pre-dried seeds were used for analysis.

protection as an antioxidant, such as in the case of *Arabidopsis* (Stevenson and Hurst, 2007; Rajjou and Debeaujon, 2008). This further supports the storability of *T. pandacaqui* seeds in seed genebanks.

On the other hand, the proximate composition of its seeds denotes high oil content of 28.7407±0.09316% (Table 4) which is higher than soybean with an oil content of around 19.56% (Eckey, 1954; Paddley *et al*., 1994). Seeds with high oil content are often observed to have lower longevity in storage among orthodox types and should be monitored for viability after five years in long-term storage conditions (Rao *et al*., 2006). Thus, although its seed is amenable to *ex-situ* conservation through seedbanking, its viability must be monitored in shorter intervals due to its oily characteristic. Conservation and management practices specific for storing oily orthodox seeds should be followed. This includes following the low-constant oven drying method in routinely determining the seed moisture content (Rao *et al*., 2006; ISTA, 2021). Oily seeds tend to have lower equilibrium moisture levels at a specific relative humidity and temperature compared to starchy seeds (Bradford, 2004). In seed viability equations, species-specific moisture content constants (C_w) in oily seeds were much lower (Hong *et al*., 1996). This means that seeds of *T. pandacaqui* will dry to lower moisture contents, at the same relative humidity and temperature, compared to other non-oily species; however, it is necessary to dry these oily seeds more to obtain the same relative increase in longevity during storage.

Conclusion and Recommendation

Tabernaemontana pandacaqui seeds have orthodox storage behavior and can be stored under low temperature and moisture content to prolong their storage life; thus, they are amendable to *ex-situ* conservation through seedbanking but their viability must be monitored in shorter intervals, at least every five years, due to their high oil content. During seedling development, it exhibits a characteristic phanerocotylar epigeal type of germination wherein the foliaceous cotyledons are raised (BBCH 09) and unfolded above the soil surface (BBCH 10).

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