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Research Article

In vitro Studies on Pollen Viability, Pollen Germination and Pollen Tube Growth of *Hornstedtia conoidea* Ridl. – a Philippine Endemic Ginger Species

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ABSTRACT

Zingiberaceous plants are predominantly propagated through underground rhizomes and seeds. In this study, the viability of pollen, rate of pollen germination and length of pollen tube of the Philippine endemic *Hornstedtia conoidea* were examined. Four petri plates containing pollen samples were prepared, of which two petri plates were used immediately after the collection, while the other two petri plates were stored for one week. The determination of pollen viability was made employing stain tests using IKI (iodine + potassium iodide) solution and safranin and measured immediately after the collection and after one week. Likewise, pollen germination test was carried out *in vitro* on agar medium and measured after 24 hours and one week of incubation. Data revealed that pollen viability was higher in the samples which were sown immediately with safranin with percentage pollen viability of 92.43%, while IKI test was lower with 89.36%. On the other hand, the pollen stored for one week measured 47.29% for safranin test and 33.14% for IKI test. The percentage germination of pollen after 24 hours was 72.65%, while 11.13% after one week. The pollen samples which were subjected for tube growth were examined for two weeks and gave positive results. Overall, it can be concluded that *H. conoidea* pollen can successfully be collected and stored for certain time. Knowledge regarding the pollen viability, pollen germination and pollen tube growth of this species will give practical benefit for plant breeding and conservation purposes, since *H. conoidea* is found in the wild, and as of now, the species has been recorded only in the Philippines.

Keywords: Ethno-medicinal uses, native species, palynology, "tagbak", Zingiberaceae

Introduction

The ginger family (Zingiberaceae) consists of herbaceous perennial plants that grow well in humid tropical and subtropical areas and are mostly distributed in Southeast Asia [1]. Furthermore, Zingiberaceous plants are animal-pollinated tropical monocotyledons and display a broad range of pollination and breeding systems [2, 3]. In the Philippines, this family is represented with 16 known genera [4]. One of these is *Hornstedtia* Retz., which is characterized by a rigid spindle-shaped inflorescence composed of many sterile bracts often with reticulate nervation [5]. *Hornstedtia conoidea* Ridl., a Philippine endemic ginger species and known for its local name *tagbak* is the most abundant Philippine *Hornstedtia* species in the Province of Bukidnon and other areas of Mindanao. Seeds from its ripe fruits are edible and eaten by the local people in the said Province and claimed to cure stomach disorders [6].

Pollen are immature endosporic male gametophytes of seed plants which produce the male gametes or sperm cell and are found in the reproductive organ of the plant which is necessary for

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the angiosperm species to reproduce sexually [7, 8]. Estimation of pollen viability is useful for plant breeders and geneticists in eliminating the time and space problems [9] and can be made using direct methods such as the induction of *in vitro* pollen germination [10, 11]. The assessment of pollen quality by its *in vitro* germination is a useful method for determining the acceptability of pollen for artificial pollination [12]. Furthermore, studies of *in vitro* pollen germination and pollen tube growth are important for understanding fertilization and seed formation in flowering plants and are very useful for explaining any lack of plant fertility [13].

The importance of pollen in reproduction for the continuity of the species must be addressed. Since *H. conoidea* is endemic to the Philippines and used by the local people as food and alternative medicine, there might be odds that an over collection of its fruits and rhizomes could decline its populations without efforts to propagate. Thus, this study was undertaken to examine its pollen viability, pollen germination and pollen tube growth to contribute understanding and to provide insights to plant physiologists, plant breeders and conservationists on the horticultural practice of its reproduction. Furthermore, the updated distribution and ethno-medicinal uses of *H. conoidea* are also provided herein.

Material and Methods

Collection of pollen samples

Fresh hermaphrodite flowers of *H. conoidea* were collected early in the morning from 20 plant individuals of the same population. The collection was done during the anthesis of the flowers at Acma's residence of Market Site of Central Mindanao University, Musuan, Bukidnon from November 2015 to February 2016. The collected flowers were placed in zip–lock cellophane bags to prevent drying and brought immediately to the laboratory. Pollen samples were collected from matured stamens and placed in four separate petri plates. Two petri plates were sown immediately with pollen for testing the presence of starch, pollen viability, pollen germination and pollen tube growth, while the other two petri plates were stored in the laboratory at a maintained temperature of 20°C and were also sown with pollen for the same tests (except for testing the presence of starch) one week after incubation.

Pollen viability

For determining the presence of starch, fresh pollen samples were mounted on glass slides and tested with a drop of IKI (iodine + potassium iodide) solution. As reported by several studies, pollen viability can be assessed by staining and direct count. Hence, another set of pollen samples for the determination of the pollen viability were prepared separately using a drop of IKI solution and safranin and enclosed with cover slips. Each slide was examined under microscope to determine counts of viable and inviable pollen. The viability test was also done after one week using the stored pollen. Pollen samples which turned brown or black were scored as fertile, whereas pollen which remained colorless or less colorful were scored as infertile. Calculation of percentage pollen viability was determined using three replicates from about 200 samples each.

In vitro pollen germination

Under natural conditions, pollen of different species often require specific media for germination [14, 15]. In this study, the germinating medium of Karni and Aloni [16] with modifications by Reddy and Kakani [17] was used since it gave positive results to the other ginger species examined by Mendez *et al*. [18]. The experiments were conducted *in vitro* conditions using agar plate method. An estimated 10 mL of germinating medium was dispensed into three sterilized petri plates. Pollen were sprinkled on the medium by brushing on each petri plate. The whole procedure was completed within 30 minutes to avoid pollen desiccation. Experiment was done in a completely randomized design (CDR) with three replicates and selected independently in the slide to avoid duplication. Pollen germination percentage and pollen tube length were measured under light microscope (100×) examined from 10 randomized selected squares after 24 h of incubation. The same procedure was done on the one-week old stored pollen. The pollen was classified as germinated if at least the beginning of a developing tube could be seen emerging from one of the pores or reach its diameter [19, 20]. The percentage of pollen germination for each petri plate was calculated [14, 21, 22]. For each reading, 200 pollen were counted and scored as germinated or ungerminated depending on whether an intact pollen tube could be seen emerging [23].

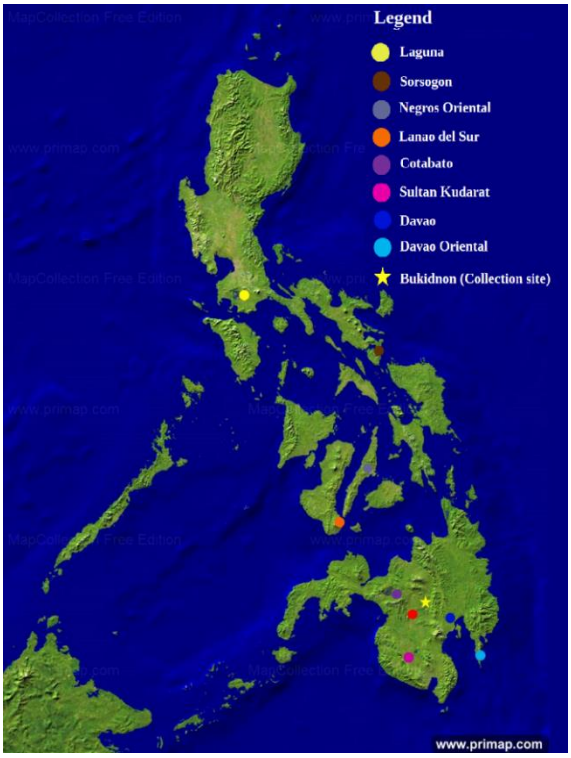

Figure 1. Map of the Philippines including the distributions and collection site of *H. conoidea* (Map source: Primap [25])

Figure 2. *H. conoidea*: Habit (a), inflorescence (b), and infructescence (c)

Pollen tube growth

Since *in vitro* germination is considered the best indicator of pollen viability [24], the pollen tube growth of *H. conoidea* was measured for a period of 2 weeks [18]. A total of three pollen per plate (nine pollen in total) were measured for every two days.The pollen was inoculated carefully from the petri plates to avoid contamination and examined under light microscope (LPO and HPO) for the measurement of tube and pollen tube lengths.

Results and Discussion

Distribution and ethno-medicinal uses

The distribution localities of *H. conoidea* in the different areas of the Philippines have rapidly increased from 2010 to present [see 4, 6] (Figure 1). However, threats such as habitat destruction, deforestation, burning of forests for farming and over collection have led to the decrease of their populations. Several populations of this species were observed by the main author in the nearby areas of Sibunga Falls in Kibawe, Bukidnon in 2014. However, during the conduct of this study to collect samples from the wild, no single population was found in said habitat. Thus, the samples utilized in this study were obtained from the ex situ conservation of Acma in 2010 (Figure 2). One reason of their depletion could be the deforestation in the area, since most trees which covered their populations were already cut down.

The uses of *H. conoidea* have long been recognized by the local people of Mindanao which they claim to cure different kinds of illnesses, such as diarrhea and stomach problems (fruits containing ripe seeds), fever and chills (rhizomes) and as condiments (leaves and rhizomes). In all field expeditions done by the authors, this plant either bore flowers and/or fruits and the reproductive parts were frequently visited by ants, which could be possible pollinator and the ripened fruits were eaten by rodents, which could be possible seed dispersal agent.

Presence of starch in the pollen

The test for the presence of starch revealed positive results, which agreed to the earlier report of Mendez *et al*. [18] that pollen of ginger species contained starch. Starchy pollen has been considered a feature of wind-pollinated (anemophilous) flowering plants, whereas insect-pollinated (entomophilous) species show a greater or lesser replacement of starch by sugar or lipids [26]. Positive starch reaction was categorized by Franchi *et al*. [27] in three color reactions - brown, blue and black. In the study of Wang *et al*. [28] on selected ginger species, starch reaction with IKI solution showed two colors (blue and black), and an unwanted phenomenon occurred with most or all pollen of *Globba racemosa* Sm., *Zingiber striola-*

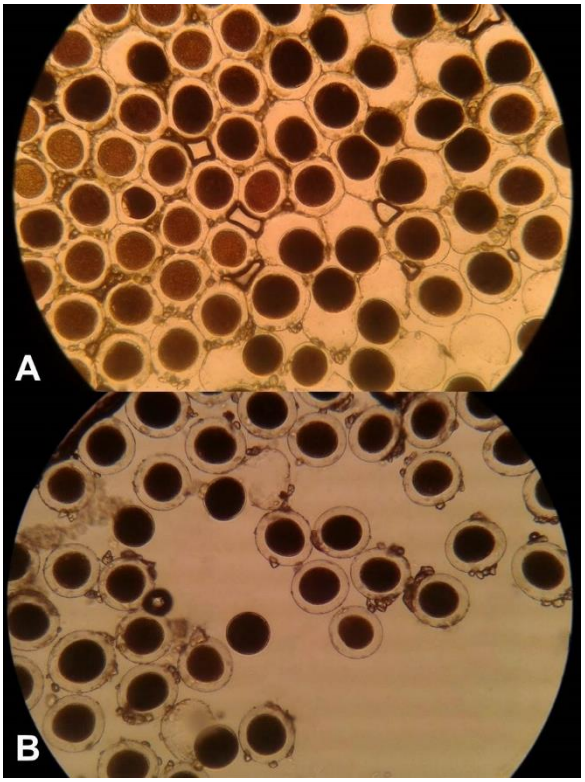

Figure 3. Pollen viability of *H. conoidea* under light microscope (100×): IKI solution (A) and afranin (B)

tum Diels., and a few pollen of *Hedychium coronarium* Koenig, *Hornstedtia flavum* and *Hornstedtia spicatum* Buch.-Ham. ex Smith stained red. On the other hand, the examined pollen of *Etlingera dalican* (Elmer) A.D.Poulsen and *Etlingera philippinensis* (Ridl.) R.M.Sm. of Mendez *et al*. [18] turned dark brown for the presence of starch.

All angiosperm pollen contains stored food reserves in the form of starch and/or lipids and can be classified in two classes: starchy and starchless [26]. Generally, the mean pollen diameter of the species with starchy pollen is significantly greater than that of the species with starchless pollen [29]. This present study, however, showed that there is no significant difference in size between starchy and starchless pollen in *H. conoidea*, although there are more variations in starchless pollen [28].

Pollen viability

The determination of pollen viability can be made using two basically different approaches namely, in-direct methods based on cytological parameters, such as color or staining pollen which are among the most reliable and widely used pollen viability tests [30, 31, 32] or direct methods, such as the induction of *in vitro* [10, 11, 33, 34, 35].

Using the test, the current study revealed that the percentage pollen viability of *H. conoidea* was higher in safranin test (92.43%) and lower in iodine test (89.36%) for the pollen which were examined right after collection. Whereas, the pollen which were stored for one week revealed lower pollen viability with 47.29% for safranin test and 33.14% for iodine test (Figure 3). Viability of pollen is extremely important for sexual reproduction of plants as reproduction success largely depends on the pollen dispersal and effective pollination within and between populations [36, 37, 38, 39]. However, pollen viability has been reported to be affected by several factors. It depends not only on its quality, but is also related to temperature, mineral nutrient and different plant growth regulators etc. in the germination environment [40]. Viability of pollen also mainly depends on relative atmospheric humidity at shedding and during pollen transport and pollen of different species need a high level of relative humidity to germinate [41-47].

Pollen germination and pollen tube growth

Pollen viability can be assessed through *in vitro* germination. Pollen of some species can germinate in water [48], while other species require more complex media for germination [12]. The media used for *in vitro* germination of pollen of different species include simple sucrose/boric acid media to complex media [49]. In earlier studies, the most widely used medium for determining *in vitro* pollen germination and tube growth were agar, sucrose (with different types and concentrations) and boron [50, 51, 52]. The sugar used in the culture medium aimed to provide a balance between the osmotic solution and pollen germination [53], while agar added to such media provide stability, so that the growth of pollen tubes can be observed [54]. There are also several advantages of using agar in germination tests, such as the ease of taking carbohydrate, creating stable relative humidity and providing aerobic conditions [12].

For germination tests, a medium should also contain some nutrients (e.g., calcium, magnesium sulfate, potassium nitrate or boric acid) [35]. *In vitro* method for pollen germination and pollen tube growth could be particularly valuable in assessing the viability of stored *H. conoidea* pollen

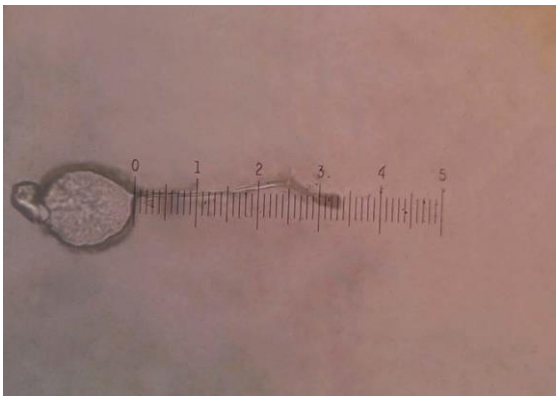

Figure 4. Pollen tube growth of *H. conoidea* under light microscope (100×)

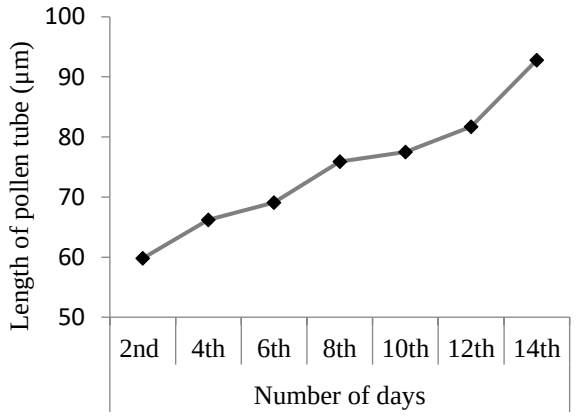

Figure 5. Length of pollen tube growth of *H. conoidea* monitored for two weeks

because *in vitro* pollen germination is a more reliable indicator of pollen viability [55, 56, 57]. *In vitro* pollen germination is also useful to detect alterations in germination or tube growth performance [58, 59, 60, 61].

In this study, the percentage germination of *H. conoidea* pollen was 72.65% after 24 hours of contact with agar medium and 11.13% after one week from initial plating (Figure 4). This study showed higher result compared to the data obtained by Mendez *et a*l. [18] for the two *Etlingera* species. The higher percentage germination, imply that the pollen has more capacity to germinate and develop. This present study and the study of Mendez *et al*. [18] also support the field observations of Acma and Mendez [62] that among *Etlingera dalican*, *Etlingera philippinensis*, and *H. conoidea*, the latter species often bore fruits, followed by *E. dalican* and rarely in *E. philippinensis*. For the pollen tube growth, it revealed that during the 2nd day of observation, the pollen tube lengths reached 58.4 – 61.2 µm, while in the 14th day, pollen tube lengths reached 94.4 – 97.2 µm (Figure 5).

It is noteworthy that there are several factors that might affect the pollen germination and pollen tube growth in the examined *H. conoidea* pollen. One of which is the temperature, the very basic factor controlling the environmental conditions and influences pollen germination and longevity in stored pollen [63, 64, 65]. The pollen germination rate is also greatly influenced by the pH of germination medium, which has also been shown in several plant species [39, 66 – 71] and relative humidity and composition of the germination medium [72, 73]. Wang *et al*. [74] also concluded that boron has a regulatory role in pollen germination and pollen tube growth in *Picea meyeri* and the inhibition of pollen germination might also be affected by aluminum [75 – 79], in which the presence of aluminum was earlier reported by Acma and Mendez [62] in *H. conoidea* pollen.

Overall, the staining techniques in this study aimed to determine pollen enzymatic activity and membrane integrity, while *in vitro* germination assays determined the actual germination ability of pollen under suitable conditions [80, 81].

Conclusion

This study assessed pollen viability, *in vitro* pollen germination and pollen tube growth of *H. conoidea*, contributing to the knowledge of its reproductive biology and offer insights for conservation management and propagation protocols. Based on the results, it is concluded that *H. conoidea* pollen successfully germinated using agar medium and the viability can be stored for certain time. Knowledge regarding this study will be useful for plant breeding and conservation purposes, as this species is found in the wild, and up to now has been found only in the Philippines.

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