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Morphology and reproductive biology of *Daiswa polyphylla* (Smith) Raf.

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Abstract *Daiswa polyphylla* (Smith) Raf. is a rare medicinal plant found in the highlands of Thailand. The research findings found that the flower emerged from the middle of a leaf whorl in April to May, bloomed from May to June, then fruit set and seed matured during May to January. The plants entered dormancy from September to January until resuming growth in April of the next growing season. Reproductive biology showed stigma receptive approximately 3-5 weeks after which it dried up. Pollen was 69.68 percent viable at anthesis and remained three percent viable at 33 days after blooming. *D. polyphylla* fruit took the time to develop approximately 9-28 weeks after flowering. Fruit ripening in 4-18 weeks were found during September to January. All seeds were viable from tetrazolium test, and seed germination from one fruit was approximately 45 percent. The research findings would possible be used for propagation and cultivation of *D. polyphylla*.

Keywords: Melanthiaceae, Pollen viability, Stigma receptivity, Seed germination

Introduction

Daiswa polyphylla (Smith) Raf. (Trias-Blasi *et al*., 2017) is a perennial plant in the Melanthiaceae family. It is considered a rare medicinal plant and grows in the highlands of northern Thailand. Its rhizomes found underground contain steroidal saponin, which are anti-cancer (Sun *et al*., 2011; Puwein *et al*., 2018; Gupta *et al*., 2021) and anti-tumor (Wang *et al*., 2006; Zhu *et al*., 2011). The plants are also used for asthma and heart ailments, analgesic, antiphlogistic, anthelmintic, antipyretic, antispasmodic, antitussive, depurative, antibacterial, antileishmanial, immunostimulating, febrifuge, tonic and narcotic, and applied as antidote to the bite of poisonous insects and snake (Quattrocchi, 2012). Moreover, its leaves contain polyphenolic compounds such as phenol, flavonoids, and tannin (Mohd *et al*., 2018). Currently, too many *D. polyphylla* (Smith) Raf. rhizomes are dug up for sale. This situation had been found in China (Cunningham *et al*., 2018; He *et al*., 2006), India (Paul *et al*., 2015; Bhat *et al*., 2017), and Nepal (Kunwar *et al*., 2020), The

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International Union for Conservation of Nature and Natural Resources (IUCN) has determined that the plant is at risk of becoming extinct (Madhu *et al*., 2010).

The plant was previously classified in genus *Paris*. Therefore, both *Daiswa* and *Paris* are used interchangeably in this document. It has been reported that there are 20 species of *Paris* (Madhu *et al*., 2010). Only two species, *P. polyphylla* var. *chinensis* and *P. polyphylla* var. *yunnanensis*, are considered very important (Sharma *et al*., 2015). However, only var. *chinensis* is found in the northern region of Thailand in provinces such as Chiang Mai, Chiang Rai, Phrae, and Nan. It grows in evergreen forests between 900 and 2,000 meters above sea level (Forest Herbarium, 2016). The National Identity Office has categorized it as a rare local herb which is worth preserving (The National Identity Office, 2007). There have been a few studies of this plant in Thailand. The objectives were to study flower morphology, reproductive biology, and growing environment of *D. polyphylla* (Smith) Raf. The information would be helpful for conservation, reproduction, and breeding of *D. polyphylla* (Smith) Raf.

Materials and methods

Plant materials

D. polyphylla (Smith) Raf. plants were planted at the Pang Ma-O Highland Development Project Via Royal Project System, Mae Na subdistrict, Chiang Dao district, Chiang Mai province, Thailand, at 1,105-1,120 meters above sea level, latitude 19^{o} 16’ 27” and longitude 98^{o} 54’ 10”. Fifty plants, which flowered from 2015-2016 were marked for all studies.

Flower morphology and anatomy of Daiswa polyphylla (Smith) Raf.

The plant structure characters included plant height, leaf size, flower diameter, sepal and petal sizes, stamen and pistil sizes, which measured from 20 plants. The anatomy of the floral parts was studied. Plant samples were collected and fixed in formalin-acetic acid-alcohol (FAA) solution. They soaked through five times in ethanol dehydration and embedded in paraffin. The sections were cut by a rotary microtome, dyed with Delafield’s hematoxylin, and observed under Olympus BX50 microscope. Pictures were taken by Olympus DP21 Microscope digital camera.

Reproductive biology of Daiswa polyphylla (Smith) Raf.

Growth and development of *D. polyphylla* (Smith) Raf. from blooming to fruit maturation were recorded every month from April to January. Fifteen flowers in the collection area were observed. Stigma

receptivity (Figure 1A and 1B) was done by testing the stigma receptivity of 15 flowers, recorded by placing wet Peroxtesmo KO paper on the stigma and recording positive receptivity when the paper turned blue (Dafni and Maués, 1998). The flowers were tested one day before blooming and blooming day. The test was repeated weekly until the stigma dried.

Pollen viability was tested by opening anther which collected from fifty blooming flowers (one anther from each flower). Pollen at anthesis (one week before blooming) was dyed with 1% acetocarmine and observed under Olympus CX31 microscope. Five hundred grains of pollen per slide (100 grains of pollen were randomly counted from five spots on slide) were observed and viability was recorded when the pollen stained red (Figure 1C). The pollen viability test was repeated every five days until none of the flowers had viable pollen. The data was described by descriptive statistics.

Seed viability was tested using the seeds which dyed in 0.1% and 1% tetrazolium solution for 24 hrs (three replications of 20 seeds). Then viability was observed and recorded when the seed tissue turned red or pink (Figure 1G). Seed germination was tested by collecting mature seeds from the mother plants and sowed in the pot with soil from the area where the mother plants grew (Figure 1H). The seeds were covered with soil approximately two centimeters depth. The pots were kept in natural conditions. Seed germination and number of seedlings were recorded at 16 months after germination (Figure 1I).

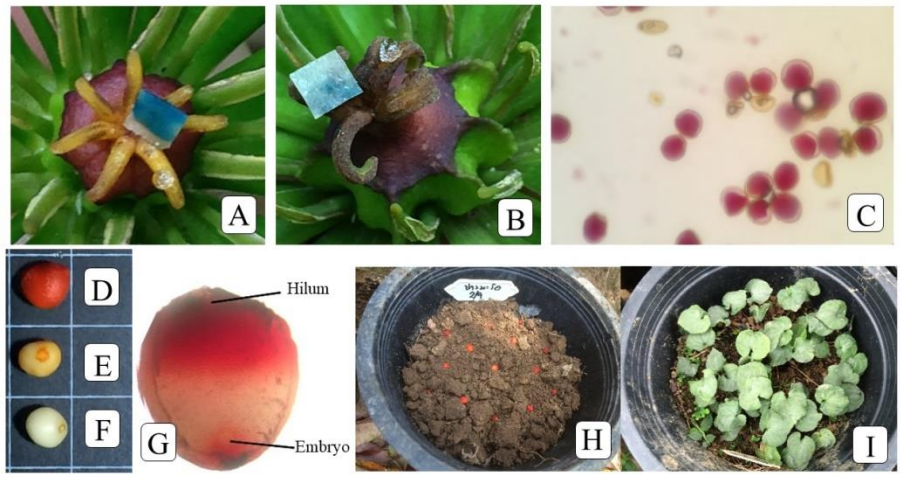


Figure 1. *Daiswa polyphylla* (Smith) Raf. in reproductive biology tests; (A) Peroxtesmo KO paper showed a color change to blue when placed on receptive stigma and (B) on dry stigma, (C) viable pollen stained red with 1% acetocarmine, (D) seed with seed coat (square=1 sq. cm), (E) seed with tegmen (red seed coat removed), (F) seed with tegmen removed, (G) viable seed stained red with tetrazolium, (H) sowed seeds before covering with soil, and (I) seedlings at 16 months after sowing

Environment planting condition of Daiswa polyphylla (Smith) Raf.

The environment in planting area including air temperature, soil temperature, relative humidity, soil water content, and light intensity were recorded by HOBO data logger from the end of April 2015 until mid January 2016.

Results

Flower morphology and anatomy of Daiswa polyphylla (Smith) Raf.

Morphology of plants and flowers of *D. polyphylla* (Smith) Raf. is shown in Figure 2. The aerial stem of *D. polyphylla* is erect. There is one main stem, unbranched, height 89.00 cm (54.50-123.00 cm), diameter 11.27 mm (7.95-16.38 mm), canopy width 43.00 cm (36.50-90.00 cm). Leaves are simple, borne 6-9 leaves in a single whorl on the stem, leaf length 23.24 cm (14.40-33.00 cm), leaf width 7.27 cm (4.65-11.00 cm), leaf thickness 0.22 mm (0.14-0.32 mm), and petiole length 4.68 cm (2.00-10.00 cm).

A single flower borne at the top of the stem from the center of the leaf whorl, flower height 22.89 cm (13.00-36.50 cm), flower width 19.80 cm (13.50-25.50 cm), pedicel length 17.77 cm (10.50-27.80 cm), pedicel diameter 4.43 mm (2.45-6.58 mm). There are 4-8 sepals or leafy bracts on the outside whorl, length 9.76 cm (6.40-13.80 cm), width 2.59 cm (1.80-3.40 cm), thickness 0.18 mm (0.13-0.25 mm). The second whorl has 3-8 petals, which reduce to a filiform structure, length 6.44 cm (4.35-9.30 cm), width 1.90 mm (0.81-2.57 mm), and thickness 0.99 mm (0.22-1.37 mm). The third whorl includes 10-22 stamens, length 1.94 cm (1.30-2.80 cm), width 1.26 mm (0.71-1.64 mm), thickness 0.73 mm (0.21-1.05 mm). The last whorl is a pistil, 1.79 cm (0.77-2.52 cm) in height with 3-8 stigma branches and 3-8 ridges of ovary surface 11.33 mm (4.36-16.20 mm) in height, ovary diameter 14.73 mm (5.8-20.36 mm).

The fruit reached maturity at 21.89 mm (14.27-36.17 mm) in height, 34.32 mm (15.14-52.13 mm) in diameter, and contains 2-38 seeds (open pollination).

Flower anatomy of *D. polyphylla* (Smith) Raf. is shown in Figure 3 which including pedicel, sepals, petal, stamen, ovary, and ovule. The pedicel is similar to the stem, and the tissue structure includes an epidermis layer, a cortex and vascular bundles. The epidermis layer is a thin wall of collenchyma cells, which form one layer without space and enclosed the pedicel and outer cover with a cuticle. The cortex covers the pedicel tissue on the inside of the epidermis layer. It is a circle of collenchyma cells with a lot of space between cells. The vascular bundles are groups of tissue dispersed in cortex tissue. They consist of xylem groups inside and phloem

groups outside, and all are enclosed by small collenchyma cells for strength (Figure 3A).

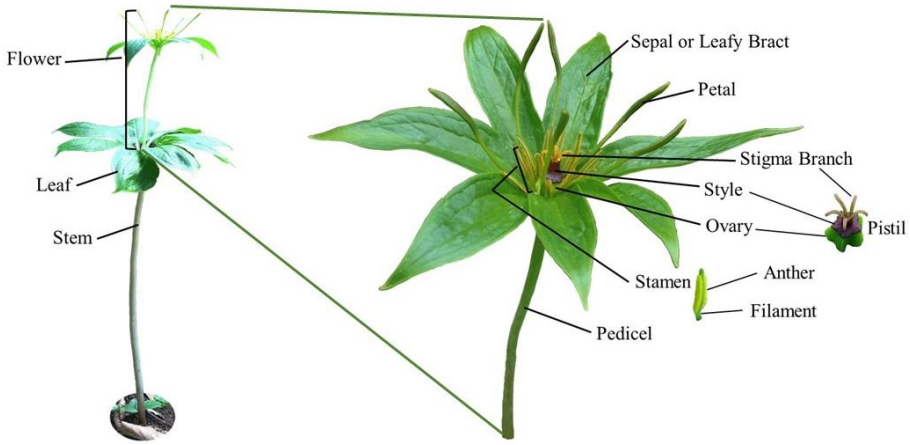


Figure 2. Plant and flower morphology of *Daiswa polyphylla* (Smith) Raf.

The cross section of sepals or leafy bracts includes an epidermis, a mesophyll, and vascular bundles. The epidermis is an outer layer that covers the internal organs. It is a thin wall of collenchyma cells, which forms a one-cell layer without space on the upper and lower sides of the sepals. Some cells change their form and role, becoming guard cells with raised stomata or stomata of the same plane. Both the upper and lower epidermis layers are covered with a cuticle. The mesophyll surrounds all of the internal sepal tissue. It has two layers of collenchyma cells, a layer of palisade mesophyll cells next to the epidermis and a layer of spongy mesophyll cells. Mesophyll cells in both layers have the same shape and there are air spaces between cells. The vascular bundles are veins that appear in the general mesophyll layer. Each bundle consists of an upper xylem group and a lower phloem group, which are enclosed by small collenchyma cells as vascular bundles. The vascular bundle region in the center of sepals is distended (Figure 3B).

The cross section of the petal included an epidermis layer, ground tissue, and vascular bundles (Figure 3C). The epidermis layer is an outer tissue surrounding the internal organs. It is thin wall of collenchyma cells which form one layer with no space. The ground tissue are alls over the petals on the inside of the epidermis layer. It is made of a circle of collenchyma cells with spaces between the cells. The vascular bundles are groups of tissue which spread in the ground tissue. They consist of xylem and phloem groups, enclosed by small collenchyma cells.

The stamen consists of a filament and an anther (Figure 3D). The filament consists of connective tissue or ground tissue and vascular bundles in the center of the stamen, both made of collenchyma cells. The anther is basifixed (present at both sides of filament). It has a cavity called a pollen

sac, which contains many round pollen grains. The pollen grains are dispersed by the opening of a suture when the anther is mature.

The ovary is at the base of the pistil of the flower. It has a single locule. Ovules adhere to the ovary wall by parietal placentation (Figure 3E). The ovule is shown in Figure 3F. The ovules are megasporangium which derived from the placenta, which grows in the ovary space. It is oval-shaped or round and attached to the placenta by a funiculus. It has two layers of integument, an outer and an inner layer. The ovule wall is not totally fused, leaving a hole at one end called micropyle, which is the entrance of the pollen tube. Ovules are amphitropous.

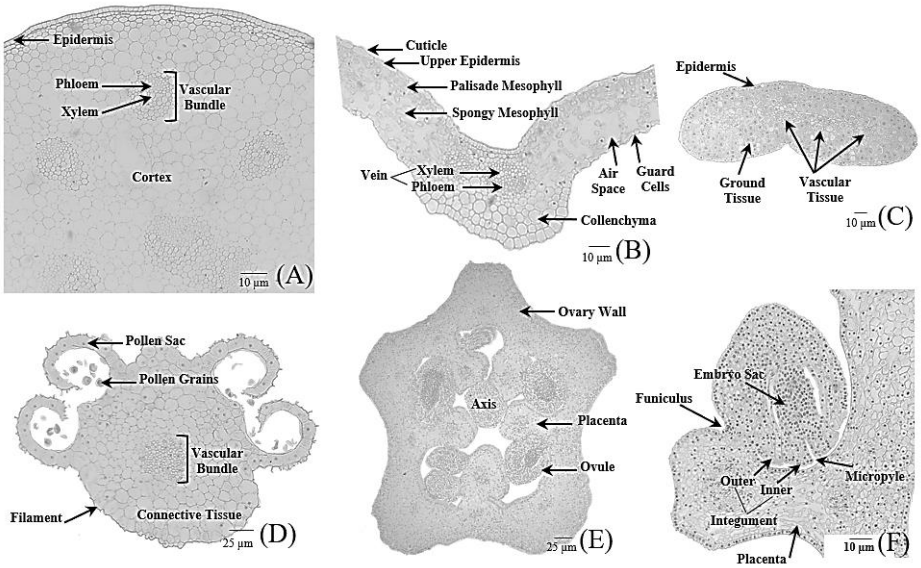


Figure 3. Organ sections of *Daiswa polyphylla* (Smith) Raf. Flower; (A) petiole, (B) sepal, (C) petal, (D) anther, (E) ovary, and (F) ovule

Reproduction of Daiswa polyphylla (Smith) Raf.

Reproduction of *D. polyphylla* (Smith) Raf. takes approximately eight to nine months (May to December or January) as shown in Table 1 and Table 2. It developed concurrently with the aerial stem growth.

Blooming showed that the flower begins to emerge while leaves are still not completely open. The flower blooms between May to June for approximately 1-4 weeks. There are two components to blooming, pollen viability and stigma receptivity. Pollen viability is concurrent with the anther opening and pollen being dispersed from one week before blooming to 38 days after blooming. The stamen dries up after all the pollen is released. Stigma receptivity is found from blooming and receptive for 3-5 weeks after it dried.

Fruit development started after fertilization. The ovary grew and developed until the fruit stop expanding which takes 9-28 weeks between May and December. Fruit ripening is found by changing color of seeds from greenish to reddish color in.4-18 weeks between September and January before fruit dehiscent.

Table 1. Reproductive development of *Daiswa polyphylla* (Smith) Raf.

Events of reproductive development	**Month of *Daiswa polyphylla* (Smith) Raf.'s development**									
	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan
Blooming		1-4 weeks								
Pollen viable		3-6 weeks								
Stigma receptive		3-5 weeks								
Fruit development		9-28 weeks								
Fruit ripening						4-18 weeks				

Table 2. Flower and fruit development of *Daiswa polyphylla* (Smith) Raf.

Events of reproductive development	Number of Samples	Weeks of reproductive development			SD
		Min	Max	Average	
Blooming peried	15	1	4	2.60	1.143
Pollen viable	50*	3	6	3.60	1.067
Stigma receptive	15	3	5	3.80	1.107
Fruit development	15	9	28	20.93	5.221
Fruit ripening	15	4	18	7.73	4.611

*samples of pollen were from flowers in the following year because the pollen were dried on the first year of study

Stigma receptivity and pollen viability tests of Daiswa polyphylla (Smith) Raf.

Stigma receptivity in *D. polyphylla* (Smith) Raf. is shown in Table 3. Peroxtesmo KO paper test resulted in a 100 to 13 percent color change to blue when placed on the receptive stigma from blooming until the stigma began to dry. The total time was six weeks from blooming.

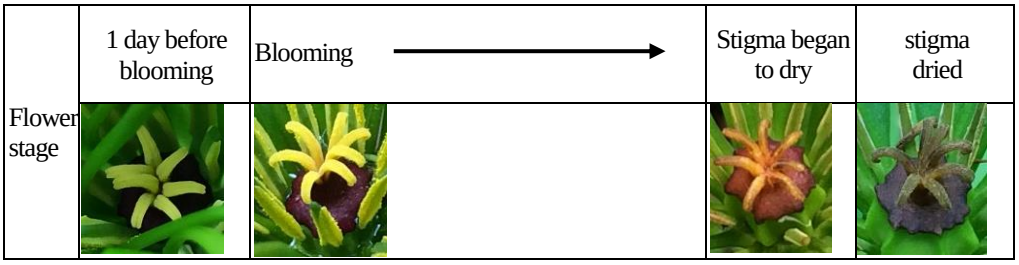
Pollen viability of *D. polyphylla* (Smith) Raf. is shown in Figure 4. All flowers had viable pollens from seven days before the flowers started to open until eight days after blooming. The percentage of flowers with viable pollens reduced to 84 on the 13^{th} day after blooming. Pollen viability reduced continuously until only two percent of the flowers had viable pollens on the 38^{th} day after blooming. The highest pollen viability was found at seven days before the flowers started opening with an average of 69.68 percent (35.00-97.00 percent) and the lowest viability was found on

the 33rd day after blooming with an average of three percent (1-6 percent) from 14 percent of all flowers which had viable pollen (Figure 4).

Table 3. Stigma receptivity in *Daiswa polyphylla* (Smith) Raf. (n=15)

	Stigma receptivity (%)						
1 day before blooming	Time (weeks after blooming)						
	0	1	2	3	4	5	6
0	100	60	33*	60	73	27	13

*There was rain on the day of observation



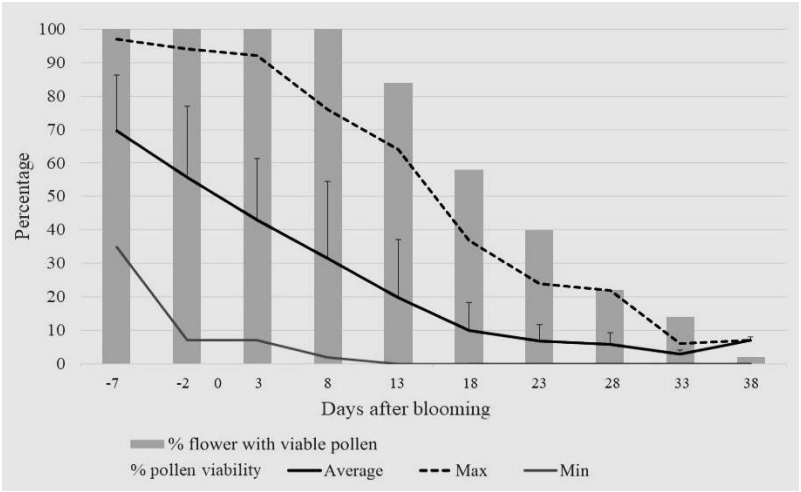


Figure 4. Percent flower with viable pollen and pollen viability of *Daiswa polyphylla* (Smith) Raf. flowers (n=50)

Seed of *D. polyphylla* (Smith) Raf. dyed with 0.1 percent and 1 percent tetrazolium solution resulted in 100 percent viability by tissue turning red. Seeds dyed in 1 percent solution were darker than seeds dyed in 0.1 percent tetrazolium solution, respective to the area of hilum, micropyle, and embryo.

Seed germination showed the total of 556 seeds form 14 mother plants, an average of 39.71 seeds (11-126 seeds) per fruit (data no shown). Seed number per fruit was higher than the record in the morphological study

because of hand pollination helped the seed set. The seeds were sowed in February, one fruit per pot. The seeds began to germinate in February of the following year, rooting and growing the first leaf, and continued to germinate until June.

Planting condition environment of Daiswa polyphylla (Smith) Raf.

The environment of planting condition of the *D. polyphylla* (Smith) Raf. were recorded. It showed an average air temperature of 21.58 ℃ (5.44-34.44 ℃), soil temperature of 22.30 ℃ (17.60-33.55 ℃), light intensity of 461.54 Lm m^{-2} (37.00-1,070.00 Lm m^{-2}), relative humidity of 81.60 percent (33.70-100.00 percent), and soil water content of 0.34 m^3 m^{-3} (0.12-0.39 m^3 m^{-3}) (Figure 5).

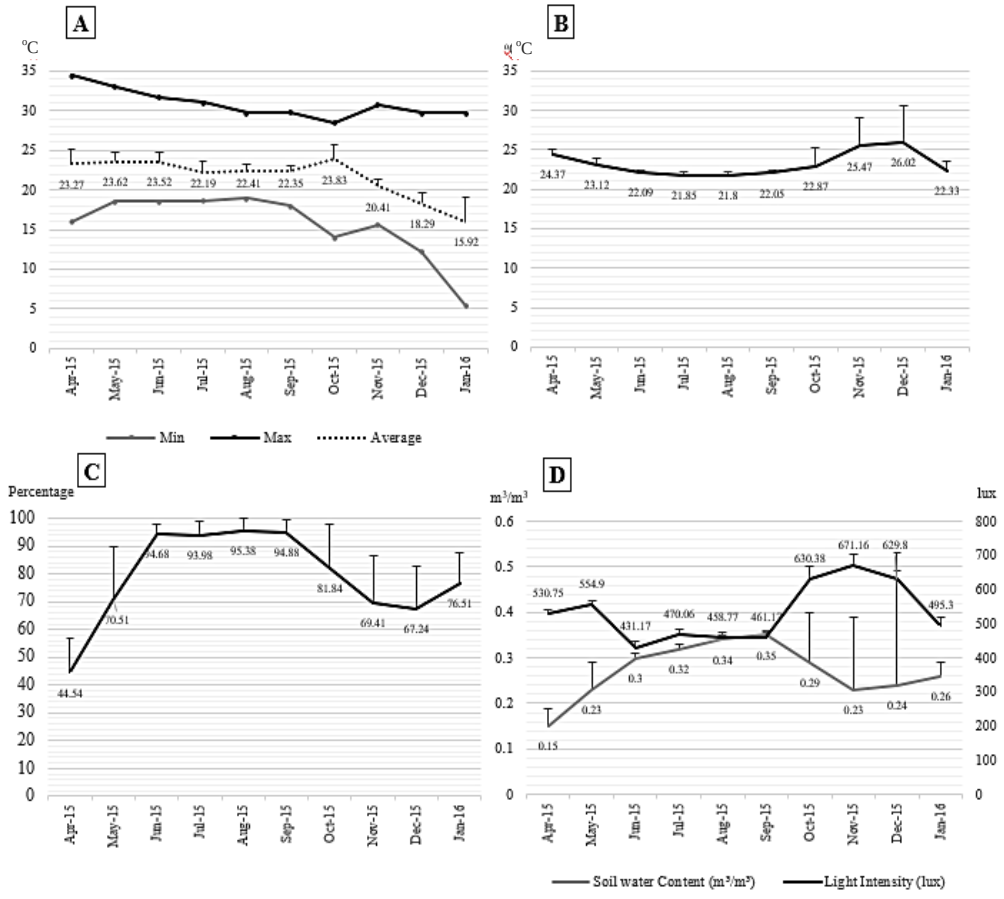


Figure 5. Monthly environmental data at a plot of *Daiswa polyphylla* (Smith) Raf. during 2015-2016 growing season; (A) air temperature, (B) soil temperature, (C) relative humidity, and (D) soil water content and light intensity

The air and the soil temperatures did not show much difference from April to October, but from November to January the air temperatures were lower than soil temperatures (Figure 5A and 5B). From June to September in rainy season, the soil temperatures were low and related to high relative humidity (over 90 percent) (Figure 5B and 5C). Soil water content was low toward the end of the cool-dry season in November to January, which related to high light intensity during these months (Figure 5D), when the plants were entering dormancy. Soil water content started to increase in May when growing season of *D. polyphylla* (Smith) Raf. began. Light intensity did not show much difference during rainy season. This is likely because the measurements were taken under the canopy of the trees where the plants were grown. It increased in cool-dry season where the leaves fall.

Discussion

Flower of *D. polyphylla* (Smith) Raf. is a single flower borne at the top of the stem in the center of the leaf whorl, comprising a pedicel, 4 to 8 sepals, 3 to 8 petals which reduce to a filiform structure, 10 to 22 stamens, and one fruit contains 2 to 38 seeds. The number and form of sepals and petals were similar to those reported in *Paris lihengiana* (Xu *et al*., 2019) and *P. viriabilis* (Yang *et al*., 2019). The petals of both species were longer than sepals, unlike *D. polyphylla* (Smith) Raf. which petals are slightly shorter than sepals. Li *et al*. (2010) showed that the number of calyces, petals, stamens, and carpels are the main factors contributing to morphological variations in different population. The flower anatomy of the *D. polyphylla* (Smith) Raf. is not very complex.

It was found that aerial stem growth above ground took place from May to January, approximately eight to nine months, and comprised five events: 1) blooming, 2) pollen viable, and 3) stigma receptive from May to June, 4) fruit development from May to December, and 5) fruit ripening from September to January. This blooming time was later than *Paris lihengiana* (Xu *et al*., 2019) and shorter than *P. viriabilis* (Yang *et al*., 2019). This difference in phenology may caused by genetics or environmental factors. *P. polyphylla* Smith in India was reported to flower during March to April, which is earlier than this study (Deb *et al*., 2015).

The stigma of *P. polyphylla* var. *yunnanensis* and *P. mairei* were highest receptive during 11-13 days and the fifth day after blooming, respectively (Wang *et al*., 2008). While report in *P. polyphylla* var. *yunnanensis* by Wang *et al*. (2013) stated the receptive period was 10-12 days after pollen dispersion. Zheng and Zhao (2012) reported that high stigma receptivity was found within only five days after pollen sac cracking.

In this study, stigma receptivity was 100 percent positive at the first week after blooming and declined gradually through the sixth week after blooming. This period of receptivity was longer than normal when compared to the other plant species (Dafni and Maués, 1998). It may be the plant's mechanism that increases the percentages of pollen germination and fruit set. In 'Nonpareil' and 'Padre' almond, pollen germination was higher in older flowers (flowers that passed full bloom and petal fall stage) than younger flowers (Yi *et al*., 2006).

Wang *et al*. (2009) reported that pollen viability of *P. polyphylla* var. *yunnanensis* was the highest on the first day of blooming at 96.5 ± 4.6 percent, then decreased to 30.1 ± 6.1 percent on the 23rd day. Wang *et al*. (2008) found that pollen could remain viable nearly the entire flowering period but decreased suddenly in the 2nd day and the 19th day, and viability lasted approximately 20-23 days. In this study, pollen was 100 percent viable for 15 days (one week before blooming to one week after blooming), then decreased to 84 and 58 percent at 13 and 18 days after blooming, respectively. Zheng and Zhao (2012) reported that pollen viability was the highest at 68.2 percent when the pollen sac cracked, then declined noticeably at the 4th day.

In teak (*Tectona grandis* Linn f.), the anthers release pollen before peak stigma receptivity, possible for most pollen to be dispersed before the papillate stigma becomes most receptive. In Eucrosia flower, pollen viability was found 1-2 days before stigma receptivity and stigma began to dry 8 days after receptive. It showed that male and female parts in the flower ready at different time (Hannantavivat, 1998). This mechanism occurs frequently in outcrossing angiosperms and is assumed to be a common method for avoidance of self-fertilization (Tangmitcharoen and Owens, 1997). The angiosperm anther dehiscence may occur before or after flower opening (Frachi *et al.,* 2007). Ashman and Schoen (1994) cited by Steinacher and Wagner (2010) reported that in insect pollinated flowers, the probability of efficient pollinator visits increases with longevity, as this increases the amount of own pollen exported and foreign pollen imported.

In this study, 64.29 percent of seeds from the fruits germinated. There was an average of 18.43 seedlings (3 to 83 seedlings) germinated from one fruit [45.71 percent (8.11 to 98.31 percent)] at 16 months after sowing. *D. polyphylla* (Smith) Raf. seeds produce a primary root about seven months after sowing and the first leaf grow about four months later, which makes one leaf stage at least 11 months after sowing (Madhu *et al*., 2010). Zhou *et al*. (2003) stated that in natural environment, *D. polyphylla* var. *yunnanensis* seeds remain dormant for a period of 18 months or longer. Madhu *et al*. (2010) stated that some seeds can remain dormant for a number of years.

The dormancy of *P. polyphylla* var. *yunnanensis* seeds was reported to be mix-dormancy (Chen *et al*., 2015). Stratification by exposure of the seeds to 4 ^{o}C 14 days and 22 ^{o}C 14 days for 16 weeks broke the dormancy, which germination was 95.3 percent (Zhou, *et al.*, 2003). Chen *et al*. (2007) reported that low temperature 0-10 ^{o}C for 2-4 months after treating the seeds at 18-20 ^{o}C for 3-4 months was suitable temperature for embryonic post-maturity and seed germination. Pu *et al*. (2016) explained that the endogenous hormone content correlated with embryo growth significantly. Increasing of GA content and GA:ABA ratio to a threshold may promote embryo development and therefore release seed dormancy.

Physical management could also improve *D. polyphylla* germination. Removing the seed coat and using plant growth regulators enhanced germination (Zhou *et al*., 2003). Covering the seedling bed with black plastic film could also improve the emergence percentage, and the seeds should be planted at suitable soil depth, 1 cm (Chen *et al*., 2007).

Planting *D. polyphylla* from the seeds takes a long time, an alternative method of vegetative propagation may be used. ShaoPing *et al*. (2008) reported that using rhizome segments reduced the duration of the pheonological phase and enhanced weight than propagation from seeds. Puwein and Thomas (2019) propagated *D. polyphylla* vegetatively from rhizome segments with bud. With the aid of plant growth regulators, BAP and NAA, sprouting was improved. Treating rhizome sections with GA_3 and IBA resulting in approximately twice sprouting and rooting percentage comparing to controls, and the best composition of planting materials was soil:loam:sand (3:2:1) (Danu *et al*., 2015). Yu *et al*. (2009) found that gibberellic acid application retarded senescence, increased rhizome yield, and increased saponin accumulation

The other alternative and effective method for propagation which will provide tremendous amount of plants is tissue culture (Raomai *et al*., 2014; Raomai *et al*., 2015). Mohd *et al*. (2021) studied vegetation pattern of *P. polyphylla* using maximum entropy modelling at 2,000-4,000 m above sea level (ASL). They found that precipitation, elevation, and vegetation type were the most important variables for predicting the habitat suitability. The information from their study can be used to plan the protection and reintroduction of the plants. This study experimented site was a plant collection plot located at 1,105-1,120 m ASL. The average air and soil temperatures of the planting area from April 2015 to January 2016 were 24.19 and 22.30 ℃, respectively. The average RH was 81.60 percent, average light intensity was 461.54 Lm m^{-2}, and average soil water content was 0.27 m^3 m^{-3}. The information from this study may contribute to better

understanding of morphology and anatomy of *D. polyphylla* (Smith) Raf. for reproductive parts with environmental condition where the plant grows.

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