CHAPTER III

MATERIALS AND METHODS

3.1 Study sites and plant materials

A comparative study of reproductive biology, including floral morphology, floral phenology, breeding system, and pollination, was conducted in two areas. *Limnophila aromatica* was studied from a cultivated plot in the Suranaree University of Technology Botanical Garden, Mueang District, Nakhon Ratchasima Province, Thailand (Figure 3.1-A). While *L. geoffrayi* was studied from natural populations in rice fields at Chum Phuang District, Nakhon Ratchasima Province, Thailand (Figure 3.1-B). Seeds of *L. geoffrayi* was collected from the study area, then stored for 0 month and 12 months for the comparative seed germination test. To study the effect of BAP on shoot generation of *L. geoffrayi in vitro*, I used the 8-week-old explants on MS medium, obtained from Plant Genetic Conservation Project Under the Royal Initiation of Her Royal Highness Princess Maha Chakri Sirindhorn, Suranaree University of Technology.

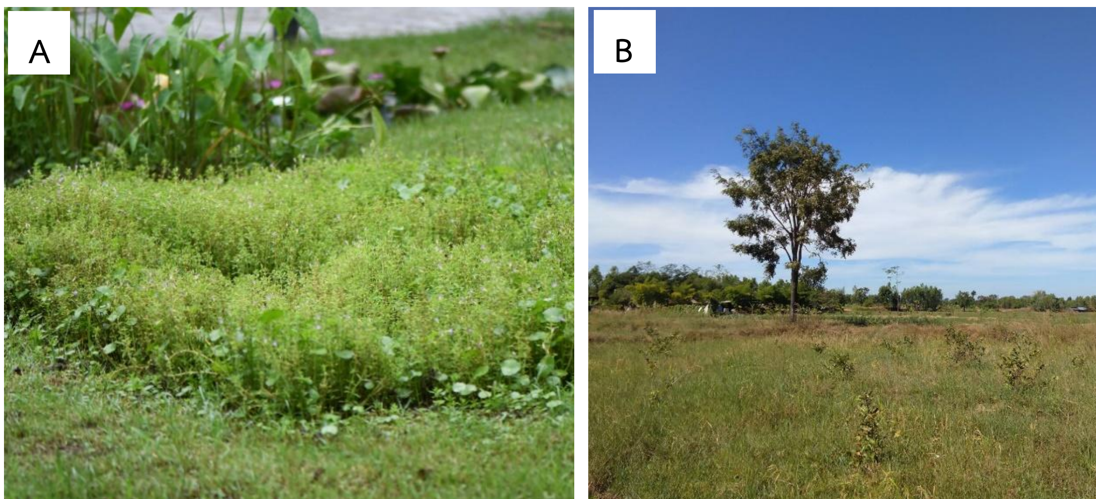

Figure 3.1 Habitat of the study sites A. cultivated plots of *Limnophila aromatica* B. *L. geoffrayi* in rice field.

3.2 Floral morphology

3.2.1 Floral morphology and Anther dehiscence

For the study of floral morphology and dehiscence stages of anthers of *L. aromatica* and *L. geoffrayi*, I collected the flowers at different stages which are the early buds (corolla lobes have not yet opened and slightly emerged from the calyx), late buds (corolla lobes has not opened yet but almost full development), and open flower (corolla tube has opened), as in Figure 3.2. Those flowers were gently dissected to reveal the position of their sex organs. In addition, five flowers of each different stages were collected and preserved in formalin-alcohol-acetic acid (FAA) solution for taking pictures of anther breaking under a stereo-microscope, Nikon SMZ645.

3.2.2 Floral phenology

The period of floral opening is interesting because it is related to the insects visiting the flower. The open floral stage of *L. aromatica* and *L. geoffrayi* is shown in Figure 3.2-A3, B3, in which the blooming flower is considered from the corolla lobes starting to spread out from each one and corolla tube. The period of floral opening was observed from a total of 40 late bud stage (from at least 15 plants) for each species. Each flower was numbered to record how many days of flowers opening. Mean blooming periods of flowers of both species was calculated.

Figure 3.2 Characteristics of *Limnophila aromatica* (A) and *L. geoffrayi* (B) at each stage of development. A1, B1. Early bud, A2, B2. Late bud, A3, B3. Open floral stage.

3.3 Breeding system

Due to the rather small size and tubular flower shape, it is impossible to do hand cross-pollination to prove the breeding system types. Thus, I studied the breeding system of *L. aromatica* and *L. geoffrayi* by conducting 3 experiments (Figure 3.3), including:

1. Autogamy: 30 floral buds were randomly covered by nylon net to check self-compatibility or the ability to pollinate without pollinating insects.
2. Emasculation: 30 late buds were emasculated by cutting off stigma and stamens to check the appearance of parthenogenesis (The ovary develops into a seed without fertilization with the male gametes) of both species.
3. Natural pollination: 30 open flowers were tracked to check for fruit set in nature.

The data were calculated as the percentage of fruiting and investigated how fruit set is affected by pollination treatment, species, and their interactions using generalized linear models (GLMs) in SPSS version 25. Type III models of Wald Chi-square statistics employing identity link functions and normal error distributions were followed subsequently. For this research, a single fruit was artificially added to the data that did not contain fruit. Using the Dunn-Sidak test, (P-values < 0.05) from multiple comparisons between treatments were adjusted (Pakum, W., Kongbangkerd, A., Srimuang, K.-O., Gale, S. W., and Watthana, S., 2023).

Figure 3.3 Different treatments in breeding system study of *Limnophila aromatica* (A) and *L. geoffrayi* (B). A1, B1. The bag experiment A2, B2. Emasculation, A3, B3. Natural pollination

3.4 Pollination

Pollination was observed in *Limnophila aromatica* and *L. geoffrayi* by randomly selecting three clumps that have the most fully bloomed flowers (approximately 30-40 flowers/clump), shown in Figure 3.4. I observed and recorded temperature, humidity, duration of insects entering each flower, species, and behavior of insects from 6:00 am to 6:00 pm during three days for at least a period of 36 hours. The insects were randomly caught using transparent plastic bags and stunned with organic compounds, ethyl acetate. I catched the insects, not more than 5 individuals of each species (This work has Certificate of approval Institutional Animal Care and Use Committee, Animal License Number U1-11459-2566). The insect samples were then put in a 95% alcohol solution, and some samples were stored in an insect box. Insect voucher specimens were identified by Assistant Professor Dr. Natapot Warrit and Dr. Prapun Triyasut, bee taxonomists from Chulalongkorn University and Ubon Ratchathani Rajabhat University.

Figure 3.4 Pollination observation of A. *Limnophila aromatica* and B. *L. geoffrayi*.

3.5 Seed germination test

To study the seed germination after storage for 12 months, seeds of *L. geoffrayi* were collected and stored for 0 month and 12 months and kept at room temperature. In selecting seeds, physical methods are used by selecting seeds that are complete, well-developed, not flattened, placed in an Eppendorf tube, and centrifuged at 12,000 g for 30 seconds. Floating seeds were removed and sunken seeds were used in the germination test. The Completely Randomized Design (CRD) in four replicates, using 25 seeds per replicate, for a total of 100 seeds for each treatment were performed.

In this study, five treatments: (1) control, without soaking (2) soaking in water for 24 hours, (3) soaking in 250 ppm gibberellic acid (GA_3) for 24 hours, (4) soaking in 500 ppm gibberellic acid (GA_3) for 24 hours, and (5) soaking in 1,000 ppm gibberellic acid (GA_3) for 24 hours (Table 3.1) were conducted. A plastic plate containing three layers of germination test paper was used to test seed germination. After sowing seeds on each plate, it was provided moisture with distilled water. All experiments were maintained at a temperature of 25 ± 2 ^{o}C in the laboratory of the School of Biology, Institute of Science, Suranaree University, Nakhon Ratchasima Province, Thailand.

For data collection, I counted the number of explants that germinated every day until 120 days. The germination success was judged by the visibility of green. Then, data were calculated using the formula as follow.

1. The percentage of seed germination (Shen, S. K., Wu, F.Q., Yang, G. S., Wang, Y. H., and Sun, W. B., 2015)

GP = (Number of seeds germinated / Total number of seeds planted) x 100

2. Germination index (Akkajit, P., and Nuamkongman, W., 2016)

GI = ∑ [(Normal number of seedlings germinated each day) / (Number of days after planting counted)]

3. Mean germination time (Labouriau, L. G., 1983)

MGT = (G1T1 + G2T2 +...+GnTn) / (G1 + G2...+Gn)

Where G: germination count on any counting period; T: time.

The results of a study on stimulating the germination of seeds with different seed preparation methods were employed one-way analysis of variance (ANOVA). Comparing the means between each experimental method with Duncan's Multiple Range Test (DMRT) at a statistical confidence level of 95 percent ($P<0.05$). SPSS version 25 was used for statistical analysis. And the effect of different storage periods, treatment series, and their interaction was analyzed using two-way analysis of variance (ANOVA).

Table 3.1 Various methods used to prepare *Limnophila geoffrayi* seeds.

Treatment	Method for preparing seeds
T_1	non-soaking (Control)
T_2	Soaking in water for 24 hours
T_3	Soaking in 250 ppm gibberellic acid (GA_3) for 24 hours
T_4	Soaking in 500 ppm gibberellic acid (GA_3) for 24 hours
T_5	Soaking in 1,000 ppm gibberellic acid (GA_3) for 24 hours

3.6 Effect of BAP on shoot generation of *L. geoffrayi*

Nodes of 8 weeks-old on MS medium of *L. geoffrayi* about 1 cm long were cut and used as the explants. They were transferred to MS medium containing 0, 0.5, 1, and 2 mg/l 6-benzylaminopurine (BAP) with 3% sucrose and 0.65% agar. The pH of the medium was adjusted to 5.7±0.1 (adapted from Dogan, M., 2019). I used 3 nodes per a 4 oz bottle. A total of 15 nodes on each MS medium containing 0, 0.5, 1, and 2 mg/l BAP were used for this experimental study.

After 8 weeks, data was recorded the average number of shoots per explant, the shoot length, and the average number of leaves per shoot. Data were analysed using the one-way ANOVA and Duncan tests were applied for post hoc tests ($P<0.05$), by SPSS progra