CHAPTER 3

Methodology

3.1 The study site

Direct seeding was carried out on a degraded site at Mon Cham, Mae Rim District, Chiang Mai (N 18° 56´ E 98° 49´, elevation 1,343). Annual rainfall (2015) was 1,324.0 mm. Rainy season normally start from May to October. The highest rainfall was found in August. Average temperature in 2015 was 21.5 °C. January was the coldest month, which had an average temperature 17.3 °C in 2015 (Figure 3.1). This area was previously used as agricultural land, but was subsequently earmarked for forest restoration by the Royal Project in 2012. Reforestation activities were funded by Plant a Tree Today Foundation in 2012 and by the Rajapruek Institute Foundation in 2013, with technical guidance from FORRU-CMU. The part of the site used for direct seeding experiments had not been planted with trees and was dominated by weeds such as *Pteridium aquilinum*, *Paspalum atratum* and *Imperata cylindrica* (Figure 3.2).

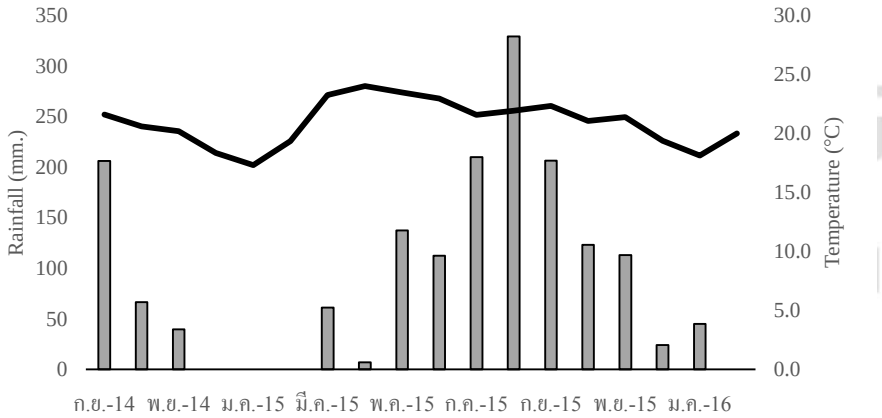

Figure 3.1 Average monthly rainfalls and temperatures at the study site, Mon Cham, Mae Rim District, Chiang Mai.

Germination and nursery experiments were carried out at the FORRU-CMU Nursery near Wat Prathat Doi Suthep.

Seed storage experiments were carried out at FORRU office in the Herbarium and Biology laboratory at Department of Biology, Chiang Mai University.

Figure 3.2 Study site at Mon Cham, Mae Rim District, Chiang Mai.

3.2 Species Selection and Seed Collection

Seeds of various native tree species were collected as they became available in every month of the year (Table 3.1) and subjected to three main experiments; immediate direct seeding in the degraded site at Mon Cham, seed storage and germination and seedling raising in the FORRU-CMU nursery.

Table 3.1 List of study species.

Species	Family	Date of seed collection	Diaspore use in this study	Storage Behaviour[3]
Acrocarpus fraxinifolius Arn.	Leguminosae	11/04/15	Seed[1]	N/A
Adenanthera microsperma Teijsm. & Binn.	Leguminosae	20/02/15	Seed[1]	N/A
Alangium kurzii Craib	Cornaceae	10/07/15	Pyrene[1]	N/A
Artocarpus lacucha Buch.-Ham.	Moraceae	01/06/15	Seed[1]	Probably Recalcitrant
Bauhinia variegata L.	Leguminosae	15/05/15	Seed[1]	Probably Orthodox
Castanopsis tribuloides (Sm.) A.DC.	Fagaceae	15/10/15	Seed[1]	Probably Recalcitrant
Choerospondias axillaris (Roxb.) B.L.Burtt & A.W.Hill	Anacardiaceae	12/07/15	Pyrene[1]	Probably Orthodox
Dimocarpus longan Lour.	Sapindaceae	01/10/14	Seed[1]	Recalcitrant
Diospyros glandulosa Lace	Ebenaceae	15/11/14	Seed[1]	N/A
Gmelina arborea Roxb.	Lamiaceae	21/05/15	Pyrene[1]	Orthodox
Horsfieldia glabra (Reinw. ex Blume) Warb.	Myristicaceae	19/05/15	Seed[1]	N/A
Hovenia dulcis Thunb.	Rhamnaceae	20/02/15	Seed[2]	Probably Orthodox
Manglietia garrettii Craib	Magnoliaceae	19/10/14	Seed[1]	N/A
Melia azedarach L.	Meliaceae	04/01/15	Seed[1]	Orthodox
Phyllanthus emblica L.	Phyllanthaceae	28/12/14	Seed[1]	Probably Orthodox
Prunus cerasoides Buch.-Ham. ex D.Don	Rosaceae	11/04/15	Pyrene[1]	Probably Orthodox
Spondias pinnata (L. f.) Kurz	Anacardiaceae	25/03/15	Pyrene[1]	N/A
Syzygium albiflorum (Duthie ex Kurz) Bahadur & R.C.Gaur	Myrtaceae	02/06/15	Seed[1]	N/A

[1] Gardner et al., 2000
[2] Kopachon et al., 1996
[3] Seed information database (SID), Royal Botanic Gardens Kew, 2017
N/A information not available

3.3 Seed Biology

3.3.1 Baseline Germination

A standard nursery germination test was carried out. Three replicates of 50 seeds were prepared in modular plastic trays with 100% forest soil. The seeds were buried about 1 cm in the media. The number of germinated seeds was counted every 7 days as well as the number of seedlings which subsequently died. Germination was defined as visual emergence of a plumule or radical through the testa. Graphs were plotted of cumulative numbers of seeds germinated and numbers of seedlings which subsequently dies vs time. Mean and variability of germination percentage and median length of dormancy (MLD) were calculated. MLD is defined as the time taken for germination of half the number of seeds that finally germinated. Germination test was monitored until 30 days after the last germination recorded.

3.3.2 Moisture Content

The moisture content (MC) test followed the ISTA rules (ISTA, 2006). Three replicates of 10 to 15 seeds were randomly selected and weighed with a digital scale accurate to $1/10{,}000^{th}$ of a gram, then dried at 103 ± 3 °C for 17 ± 1 h, in hot air oven. Seed moisture content was calculated on a fresh weight basis (Schmidt, 2007).

$$\text{Moisture content (\%)} = \frac{(\text{Wet weight} - \text{Dry weight}) \times 100}{\text{Wet weight}}$$

3.4 Seed Storage

3.4.1 Seed Storage Behaviour

Seed storage behaviour was tested following the methods of Hong and Ellis (1996). The initial moisture content of seeds was determined and the moisture content was reduced to 10% MC. The germination was tested. Seed moisture content could then be reduced 5 % and the seeds stored at room temperature or -20 °C and germination was tested again. Seeds were separated into endocarp or testa and embryo or endosperm parts and were dried under the above conditions. The dry weight of the covering parts and embryo were used to calculated seed coat ratio and the probability of the sensitivity of the seeds to desiccation.

3.4.2 Seed Storage Design

Longevity under storage was determined, using seeds stored in hermetically sealed polyethylene bags under various conditions; i) at initial seed moisture content or ii) reduced to 5% moisture content either under ambient conditions or in a refrigerator (4 °C). Seed germination tests were then carried out on three replicates of 30 seeds and monitored every 1, 3, 6, and 12 month(s) (Figure 3.3).

3.4.3 Statistical Analysis

Differences in mean percent seed germination and MLD (days) among storage treatments and species were tested with ANOVA, followed by pair-wise t-tests, when indicated. Binomial data, such as percent germination, were arcsine-transformed before analysis.

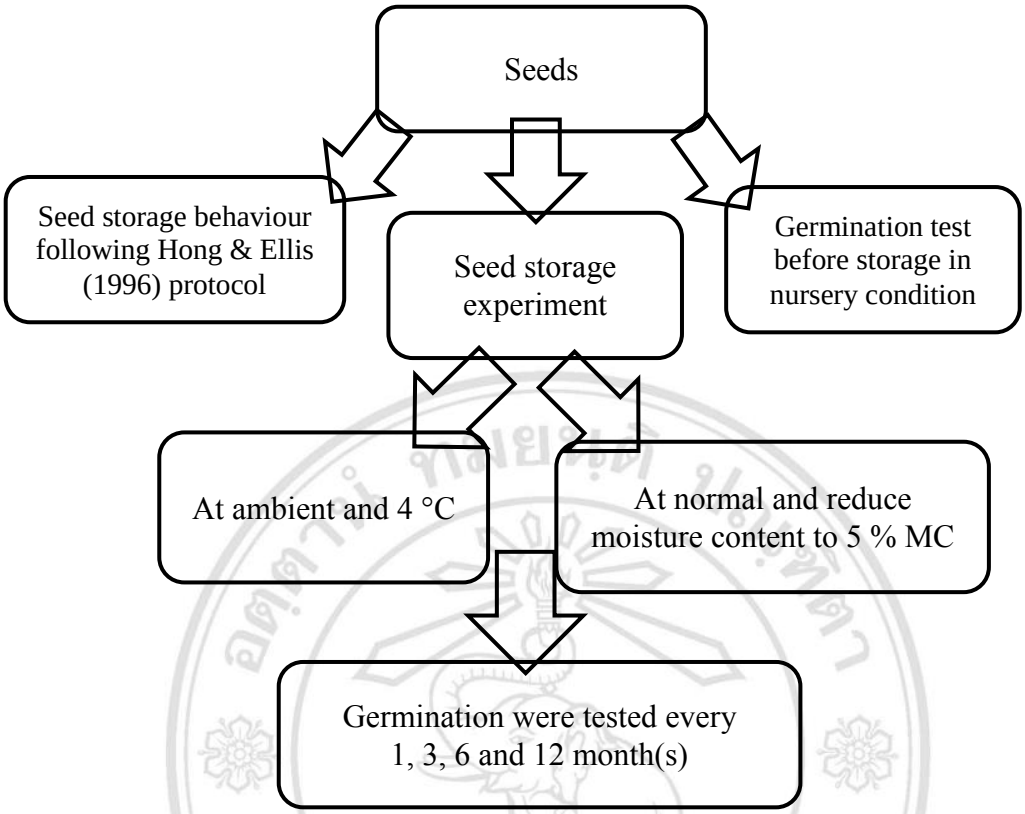

Figure 3.3 Diagram of seed storage experiment.

3.5 Field Trial

3.5.1 Direct Seeding

Seeds of native species were sown in the study site, immediately after seed collection. Seeds were positioned 50 cm apart and buried as three replicates of 50 seeds each, with at least 20 meters between each replicate. A PVC pipe was placed around every seed sown to prevent seed movement and to make the seeds easier to find for future measurements. Stored seeds were sown beside immediately sown seeds at the beginning of rainy season (12th June 2015) in a paired experimental design. Seeds of *A. kurzii* (29th July 2015) and *S. axillaris* (15th July 2015) were sown later, because they fruited during the rainy season. Seed germination was monitored weekly, until germination ceased and MLD subsequently calculated. In addition, height, root collar diameter (RCD) and crown width of surviving seedlings were monitored at beginning of first rainy season (July, 2015) and

after first rainy season (December, 2015) and beginning of second rainy season (July, 2016) (Figure 3.4).

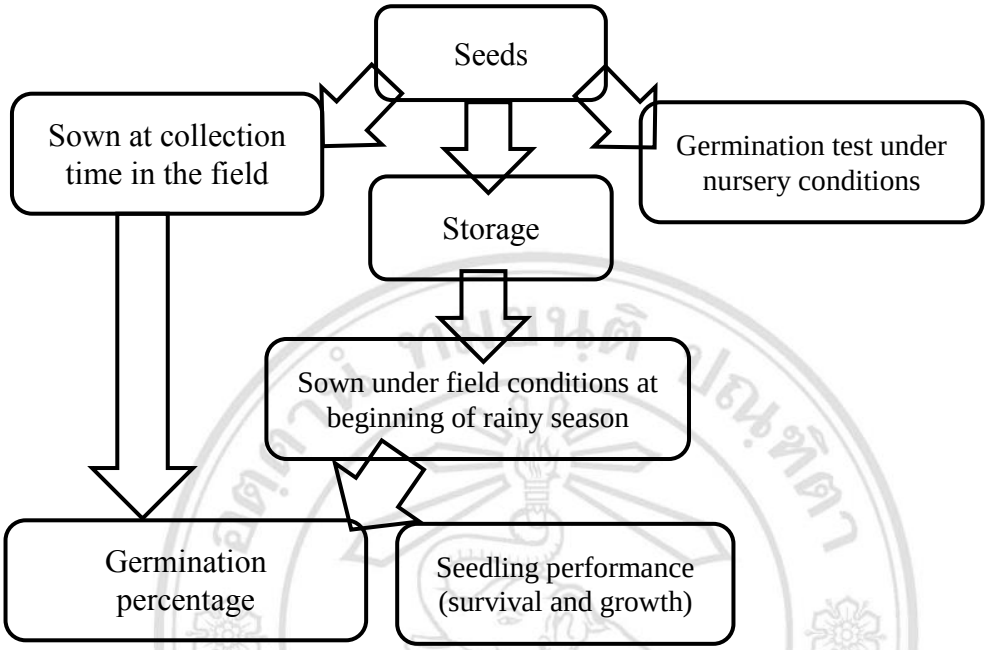

Figure 3.4 Diagram of direct seeding experiment.

3.5.2 Direct-seeded Seedlings vs Nursery-raised Seedlings

Seedlings, raised in the nursery for about 1 year, under standard nursery conditions and approximately 30-50 cm tall, were planted next to seedlings that had establish in the field in the direct seeding experiments, in a pair-wise experiment. Seedling Survival and growth of both nursery-raised and direct seeded seedlings were monitored and compared using paired t-tests. Weeds on the study site were controlled at the beginning and late rainy season, summer and winter season. Fertilizer was applied to seedlings following the recommendations of FORRU (2006).

Seedling relative growth rate (RGR) was calculated using follow equation:

$$RGR = \frac{\ln FS - \ln IS \times 36{,}500}{\text{No. days between measurements}}$$

Where in ln FS was natural logarithm of final sapling growth and ln IS was natural logarithm of initial sapling growth (Elliott et al., 2013).

Sturdiness quotient was calculated using follow equation (Elliott et al., 2013):

$$\text{Sturdiness quotient} = \frac{\text{Height (cm)}}{\text{RCD (mm)}}$$

A Relative Species Performance Index was calculated by three different methods:
i) Index was calculated from mean percent yield multiplied by absolute seedling height (cm) after one year. The highest score was ranked as 100 and the others expressed as a percentage of the highest score (Tunjai and Elliott, 2012) as following equation:

$$\text{Row score} = \%\ \text{Yield} \times \text{Height (cm)},$$

$$\text{Index} = \frac{\text{Row score} \times 100}{\text{Highest row score}}$$

ii) The calculation method was modified from Tunjai and Elliott (2012) by using height RGR (%/year) instead of absolute height as follows:

$$\text{Raw score} = \%\ \text{Yield} \times \text{Height RGR (\%/year)},$$

$$\text{Index} = \frac{\text{Raw score} \times 100}{\text{Highest raw score}}$$

iii) An index was devised which combined both survival and growth into a single indicator. The index was calculated from the relative yield, combined with relative growth index, based on seedling volume and crown width. Species values were ranked in declining order of performance.

$$\text{Growth index} = (1/3\ \pi \times r^2 \times H) + \text{RCR Crown width},$$

Where RGR is root collar diameter divided by 2 and H = RGR height

$$\text{Index} = \frac{\text{Relative yield + Relative growth index}}{2}$$

3.5.3 Statistical Analysis

Mean percent seed germination, MLD (days) both in the field and in the nursery at seed collection time and after storage, were compared using ANOVA and t-tests for multiple and pair-wise comparisons among species and treatments respectively. Binomial data, such as percent germination, survival and yield, were arcsine-transformed before analysis. Differences in growth parameters, both absolute numbers and relative values were also compared, using ANOVA, followed by t-tests. Correlation analysis was performed to determine relationships between mean absolute growth parameters (height crown width and RCD) and relative growth rates (height crown width and RCD). In addition, mean relative growth rate (height, crown width and RCD) of direct-seeded seedlings and nursery-raised seedlings were tested with paired t-tests.

3.6 Hydrogel Experiment

In July 2015, seeds of six native tree species (*Acrocarpus fraxinifolius, Artocarpus lacucha, Choerospondias axillaris, Gmelina arborea, Phyllanthus emblica,* and *Prunus cerasoides*) were sown into five media treatments, including 100% forest soil, mixtures of forest soil and 10, 20 and 30 % polyacrylamide gel or hydrogel $(C_3H_5NO)_n$ and a half-layer of hydrogel and forest soil (Figure 3.5). Seeds were sown in 2-inch diameter PVC pipes, 30 seeds per treatment per replicate (150 seeds per species per replicate) and three replicates were placed across the degraded site at Mon Cham. The field results were compared with the results of nursery germination tests (also three replicates of 30 seeds each in modular germination trays, in July 2015). Weekly monitoring of seed germination was continued until no further germination had been recorded for 4 consecutive weeks. In the field, in December 2015, baseline measurements of seedling height, root collar diameter (RCD) and crown width of surviving seedlings were made and the measurements were repeated in July 2016 (to calculate RGR). Seedling growth was not measured for the seedlings which germinated in the nursery.

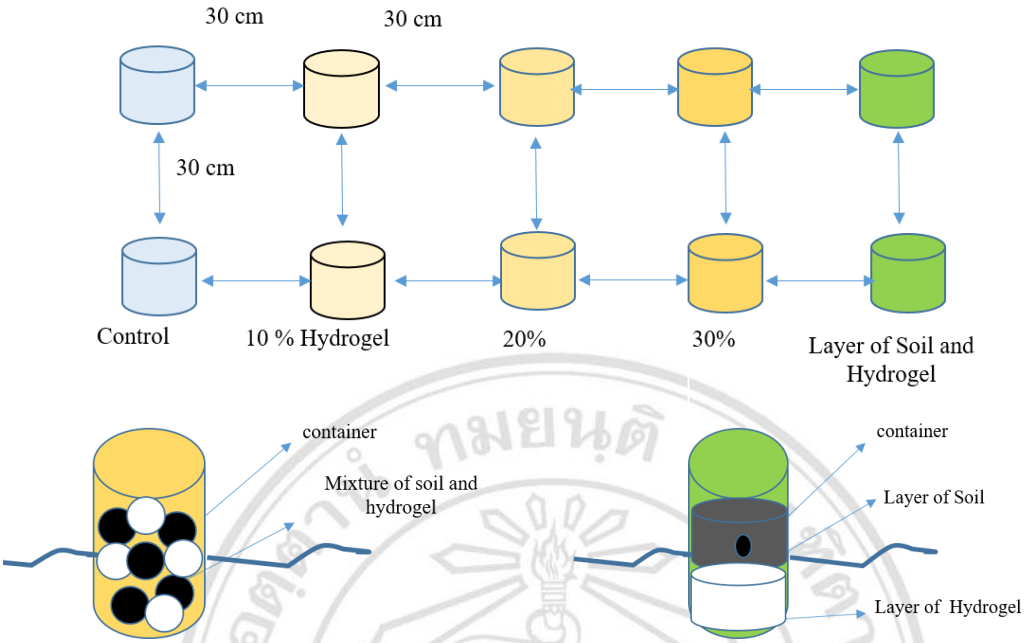

Figure 3.5 Diagram of hydrogel experiment.

3.6.1 Statistical Analysis

Differences in mean percent seed germination and MLD (days) between sites (nursery and field) were tested with t-tests. Mean differences in germination, MLD, survival, yield and growth between hydrogel treatments were identified with ANOVA followed by *post-hoc* analyses, using Tukey's HSD at α=0.05. Binomial data (percent germination, survival and yield) were arcsine-transformed before analyses. Species performance indices were calculated as described above.

3.7 Fertilizer Experiment

Experiments were performed in the nursery on saplings of eight indigenous forest tree species: *Acrocarpus fraxinifolius, Adenanthera microsperma, Artocarpus lacucha, Hovenia dulcis, Horsfieldia glabra* and *Phyllanthus emblica, Prunus cerasoides* and *Syzygium albiflora*. Seeds were germinated in modular germination trays with 100% forest soil. Seedlings with at least two pairs of true leaves were then transferred into black polyethylene bags (9 x 2 ½ inches). A mix of forest soil, coconut husk and peanut husk (2:1:1) was used as the standard potting medium (FORRU, 2006; Elliott et al., 2013).

Seedlings were prepared at least two weeks before starting the experiment to take account of mortality due to transplantation stress, so only healthy seedlings were used in the study.

3.7.1 Experimental Design

To quantify the effectiveness of fertilizer on seedling growth performance and determine nutrient allocation within the plants, the saplings were tested with three fertilizer treatments in a randomized completed block design experiment. The effects of Osmocote (FORRU's standard fertilizer treatment) and a new fertilizer developed by The National Nanotechnology Center (NANOTEC, hereafter referred to as NF). Both are slow release fertilizers, with nitrogen, phosphorus and potassium at 13, 13, and 13 % respectively. However, NF differs from Osmocote in that it has a nanocomposite coating; an alkyd resin, containing modified montmorillonite clay (mMMT), which, combined with a hydrophobic polymer layer, decelerates the solubility of fertilizer within, thus delivering a more even supply of nutrients to the plants and reducing nutrient wastage (Sitthisuwannakul1 et al., 2014). Osmocote (0.3 gram) was used as the control, since this is the standard protocol used by FORRU-CMU. It was tested against two dose sizes of NF, (0.15 and 0.3 g).

Seedlings were arranged in 3 blocks, each containing the two NF treatments and one Osmocote control. Within each replicate, seedlings were arranged in squares of 5 x 5 seedlings, within which 3 x 3 seedlings were used as the test seedlings, with the outliers forming a "guard row", to control for seedling position and buffering against external factors (Figure 3.6). So, 225 seedlings were used to form all three blocks, of which 81 were the test plants. In order to quantify soil nutrient availability, one extra block was set up with only media and fertilizer (Figure 3.7).

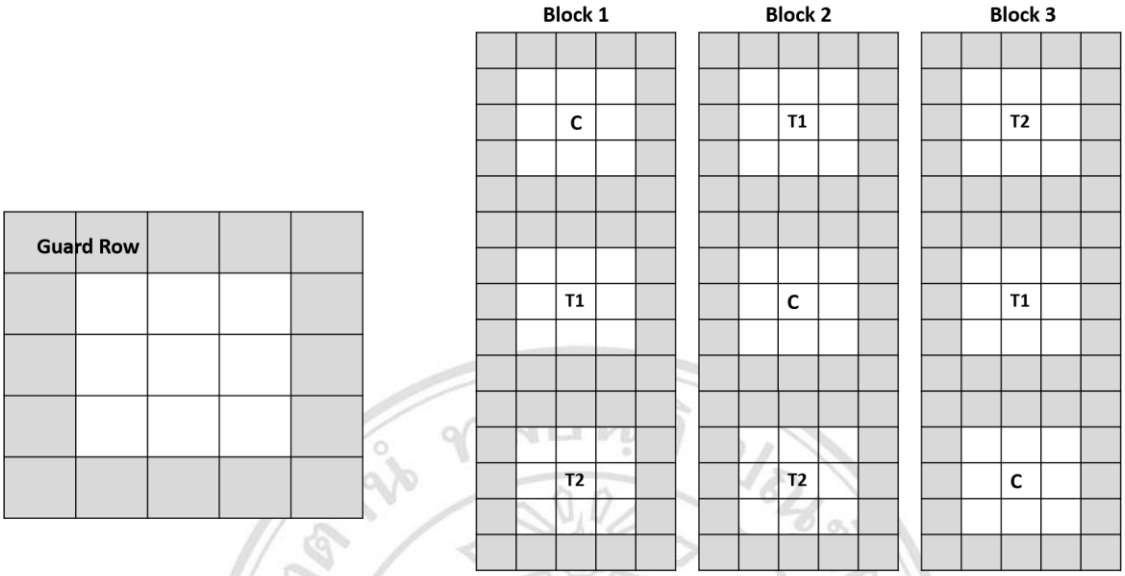

Figure 3.6 Fertilizer experimental design.

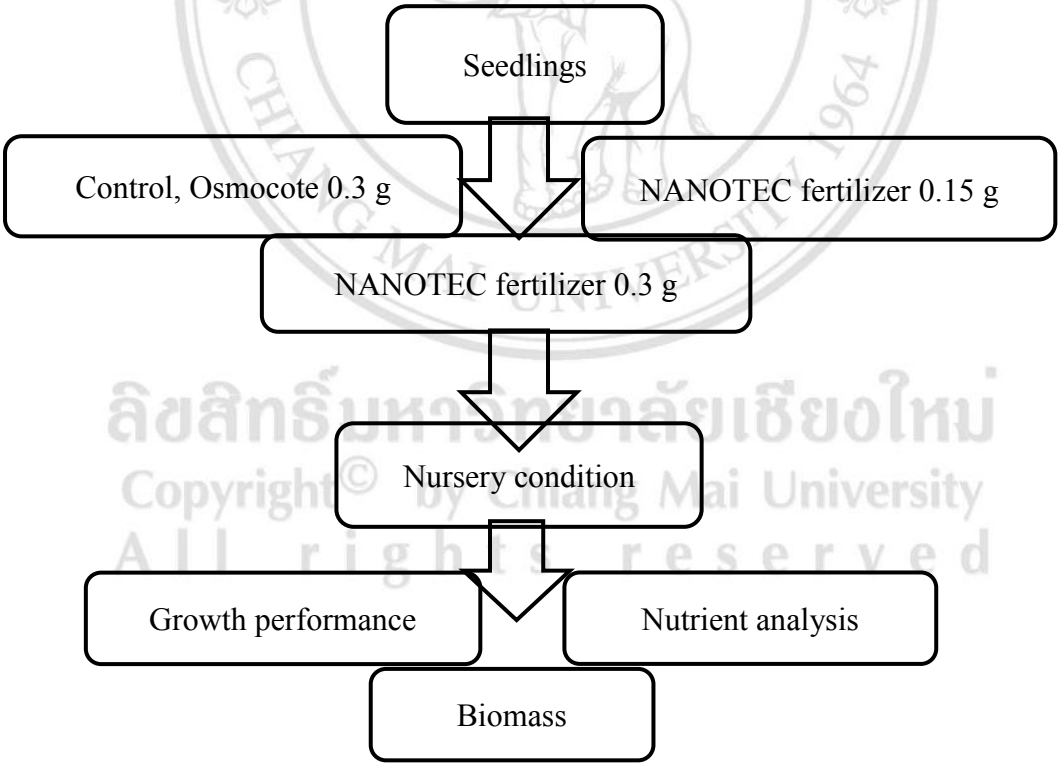

Figure 3.7 Diagram of fertilizer experiment.

3.7.2 Fertilizer Analysis

Nutrients availability in the different treatments were analyzed at the start of the experiment and at 56 and 112 days respectively. Available nitrogen, phosphorus and potassium were compared among the treatments. The media were sampled from at least one pot from each treatment and block for each species. So at least three samples were tested for each treatment and species. Furthermore, the medium from an extra block (media with only fertilizer excluded seedling) was also analyzed in order to remove the effects of nutrient uptake by the plants, thus allowing a crude estimate of nutrient uptake to be made. All samples were analyzed at central laboratory of Department of Plant Science and Soil Science, Faculty of Agriculture, CMU.

3.7.3 Seedling Growth Performance

The following variables were measured for all test seedlings: root collar diameter, crown width (at widest point), height, and health (on a scale 0-3) (FORRU, 2006). Root: shoot ratio was also determined as below equation at the beginning of the experiment and after 56, 112 and 187 days respectively.

$$\text{Root: shoot ratio} = \frac{\text{Root dry weight (g)}}{\text{Shoot dry weight (g)}}$$

Seedlings were randomly selected from each treatment and block, thoroughly removed and roots and shoots separated and weighed, dried (at 70°C until constant weight) and then weighed again.

3.7.4 Statistical Analysis

Mean relative growth rate (height, crown width and RGR), seedling biomass, root-shoot ratio and fertilizer reaming were compared with ANOVA. The differences between pairs were identified by Tukey's HSD at α=0.05.