CHAPTER 3

Methods

3.1 Study site

Field site

The study site was at Nong Hoi Royal Project Foundation (Mon-Cham) degraded area (18°56'19"N 98°49'15"E, at about 1,296 m above sea level), in Doi Suthep-Pui National Park, Chiang Mai, Northern of Thailand (Figure 3.1). The average annual precipitation from January 2015 to December 2016 was 1,419 mm with a dry season from December to April. The average annual temperature and humidity were 22ºC and 75.4%, respectively (Figure 3.2). The study site was 650 m^2 in area and 70 m away from the nearest natural forest. The degraded area was previously used as agricultural land with intensive chemicals for growing crops such as cabbage. The ground herbaceous plants were dominated by Bracken fern (*Pteridium aquilinum*), Cogon grass (*Imperata cylindrical*) and Green panic grass (*Panicum maximum*) (Figure 3.3). Recently this area was reserved for forest restoration by the Royal Project in 2012 with technical guidance from FORRU-CMU[1].

[1] Chiang Mai University's Forest Restoration Research Unit

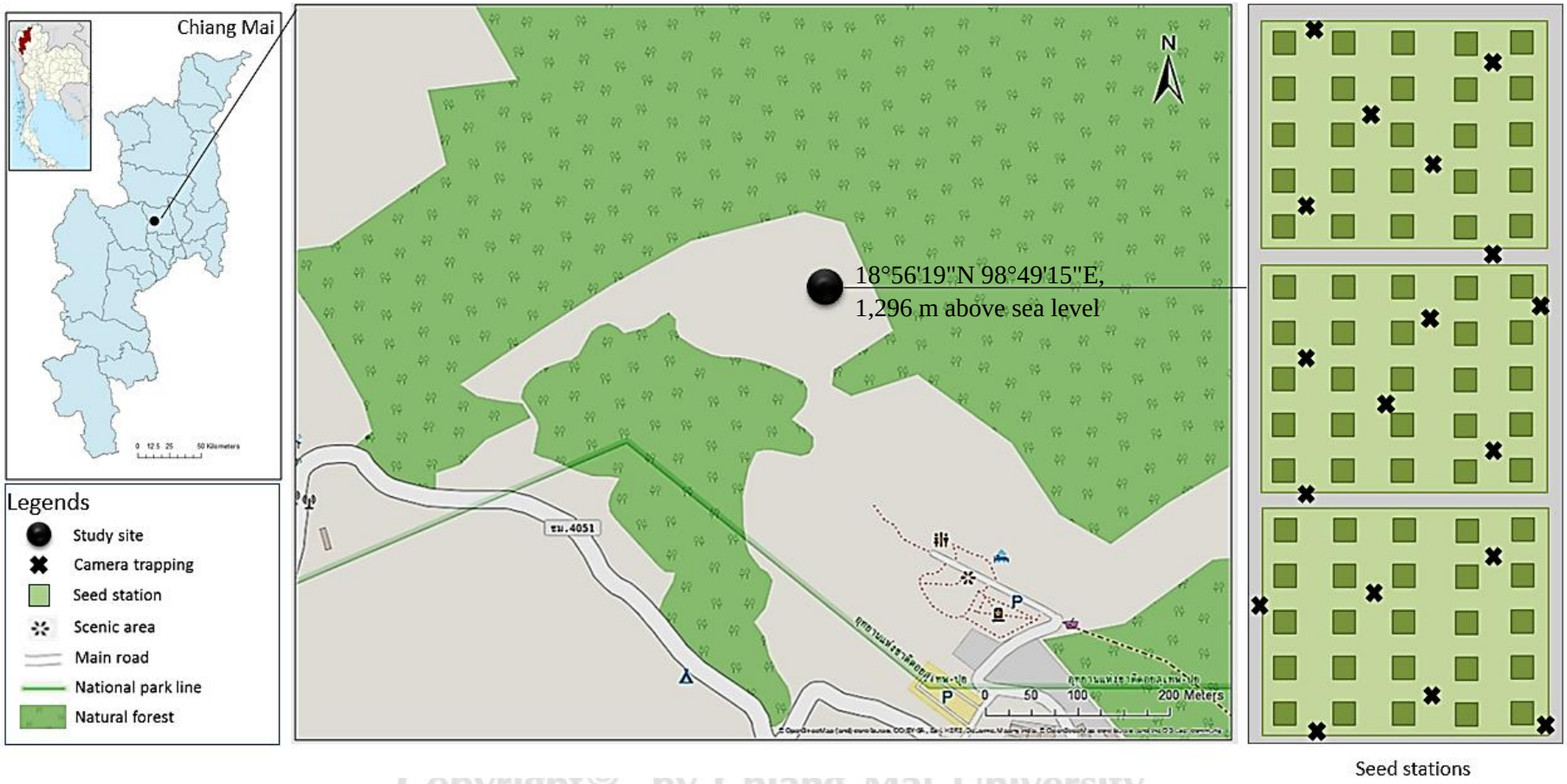

Figure 3.1 The location of study site (black circle) Mon-Cham degraded area in Ban Nong Hoi, Mae Rim, Chiang Mai, Thailand (inset map top left).

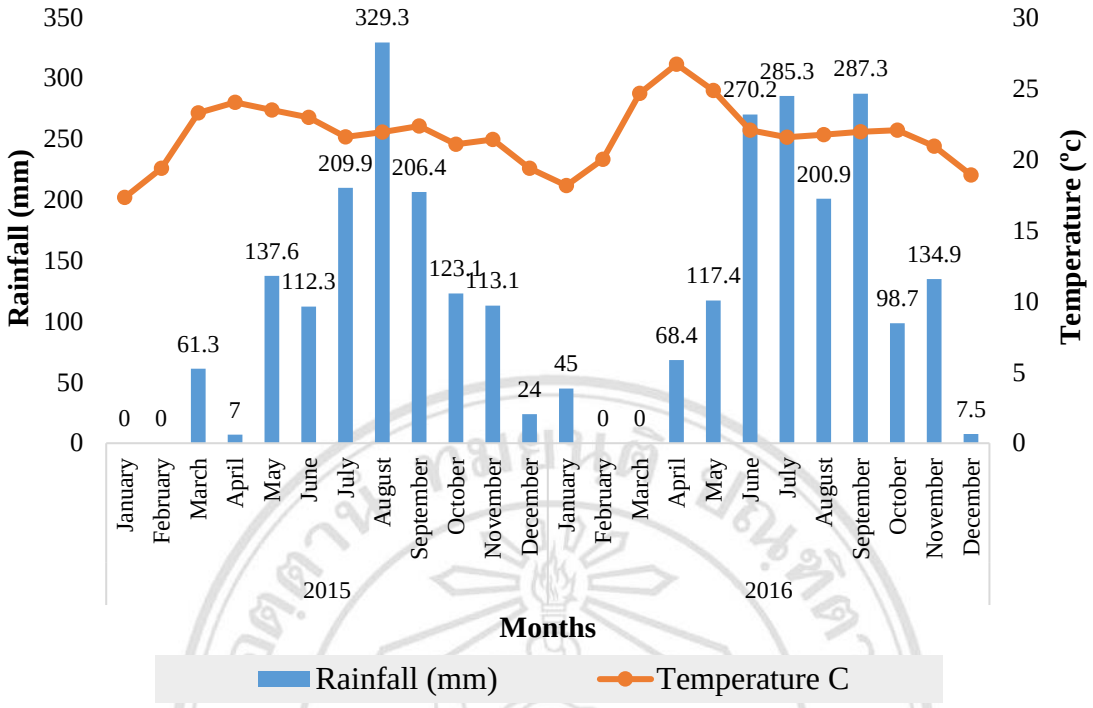

Figure 3.2 Climatic data of Ban Nong Hoi (Mon Cham) from January 2015 to December 2016. Graph showed amount of rainfall precipitation (, mm per month) and average temperature in each month (, ºC) (Meteorological Department of Thailand, 2015).

Figure 3.3 Study site at Mon Cham, Mae Rim District, Chiang Mai province.

The nursery

Seed germination tests for this study were conducted at FORRU's research nursery (Figure 3.4), located in Doi Suthep-Pui National Park (18°48'3.7" N 98°54'59.6" E, at about 1,000 meters from sea level). Tree seedlings were looked after and watered by FORRU's staff.

Figure 3.4 the FORRU's nursery in Doi Suthep-Pui National Park

3.2 Tree species studied

Five tree species were selected for experiments: *Hovenia dulcis*, *Alangium kurzii*, *Prunus cerasoides*, *Choerospondias axillaris* and *Horsfieldia glabra* (more details about these species are provided in Appendix A). All studied species were native to hill evergreen forest near the field study site (above 1,000 meter in elevation) (FORRU, 2006) in Doi Suthep-Pui National Park, Chiang Mai, Northern of Thailand (Table 3.1 and Figure 3.4).

Propagules, either seeds or (in the case of *Prunus cerasoides* and *Choerospondias axillaris*) pyrenes were collected early in the rainy season, from May to July 2015 (Table 3.1). At least 600 propagules were collected from five mother trees of each species, mixed, cleaned, dried, and stored at room temperature, until they were used in experiments.

Figure 3.5 Propagules of five tree species from smallest to the largest; *Hovenia dulcis* (A), *Alangium kurzii* (B), *Prunus cerasoides* (C), *Choerospondias axillaris* (D) and *Horsfieldia glabra* (E).

Table 3.1 Information about the five native tree species were used in this study

Species name	Seed behavior	Seed (propagule) type	Seed dry weight (g)	Seed volume (mm^3)	*Germination rate
Hovenia dulcis (RHAMNACEAE)	Orthodox	Hard-two layer of seed coat with 1 seed/locule	0.024 ± 0.000	47.38 ± 1.34	60-70%
Alangium kurzii (CORNACEAE)	Orthodox	Pyrene with 1 seed/locule	0.179 ± 0.027	392.27 ± 11.22	10-92%
Prunus cerasoides (ROSACEAE)	Orthodox	Pyrene with 1 seed/locule	0.287 ± 0.034	424.94 ± 7.89	76%
Choerospondias axillaris (ANACARDIACEAE)	Orthodox	Pyrene with 5 seeds/locules	2.602 ± 0.320	3575.19 ± 125.16	43%
Horsfieldia glabra (MYRISTICACEAE)	Recalcitrant	Soft seed coat with 1 seeds/locule	4.247 ± 0.664	7507.25 ± 322.89	94%

*Data from FORRU (2006)

3.3 Predator exclusion experiments

3.3.1 Experimental plots and predator exclusion experiments

To determine the effects of seed predators on seed removal and seed germination, I used predator exclusion experiments (Figure 3.6 and Figure 3.7). There were five treatments: -

T1: Wire cage, to protect seeds and seedlings from vertebrates (CA),

T2: Insecticide only, to protect seeds and seedlings from invertebrates (IN),

T3: Wire cage plus insecticide, to protect seeds and seedlings from vertebrates and invertebrates (IC),

T4: Open cage to control for the presence of cage (OC), and

T5: Control with no protection, no treatment applied and exposed to invertebrates and vertebrates (CO).

Figure 3.6 The exclusion experiments were established in the study site.

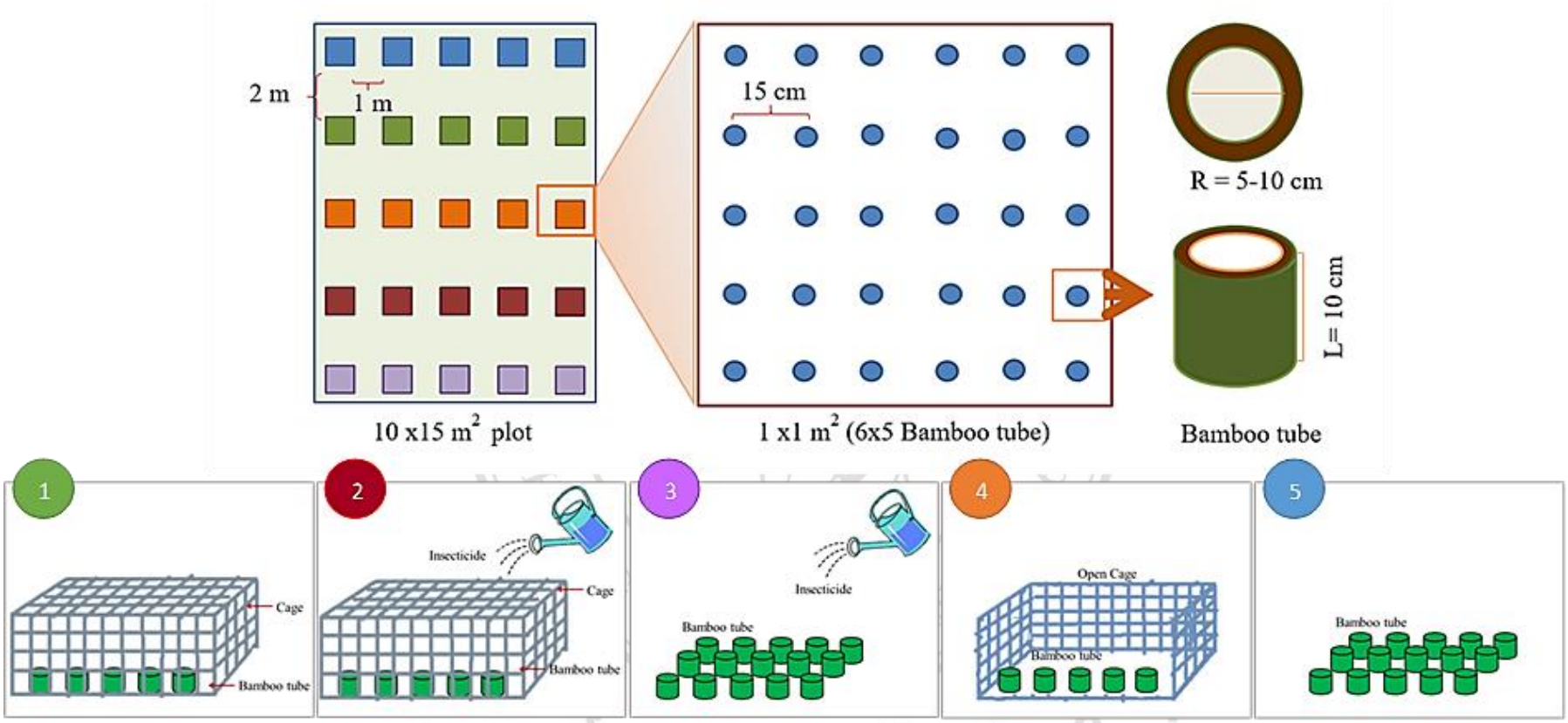

Figure 3.7 Arrangement of predator exclusion experiments in one replicate of 10 x 15 m^2 plot. There were 30 bamboo tubes in each subplot (1 x 1 m^2). One seed was sown in each bamboo tube in each subplot. Below, the plot arrangement for the five predator exclusion treatments.

Cages were built using bamboos and steel wire. The open cage was made in the same fashion as the wire cage but the top and one lateral side of the cage was removed. The insecticides Chlorpyrifos (Trade name: Kino505: Appendix D) were spayed every week from seed sowing in July until December, after all seedlings had emerged. The insecticide was mixed with water in the ratio of 2.5 ml of insecticide per 1 liter of water in a pressure spray. For each species, the 200 ml of the insecticide mixture was sprayed homogenously onto each replicate of the insecticide and the insecticide plus cage treatment.

At the Mon-Cham site, three 10 x 15 m^2 experimental plots were established in July 2015 in the middle of rainy season. The three plots were about 5 m from each other and each plot (a block) contained one of each treatment. Each plot was divided into five rows, two m apart, to accommodate five treatments. In each row, five 1 x 1 m^2 sub-plots were established to accommodate the 5 species studied. In each sub-plot, 30 bamboo tubes were buried to 5 cm deep into the soil (Figure 3.7). In each tube, one seed was sown on the soil about 1 cm deep and covered by soil (all seeds were buried).

Two treatments are applied during the nursery experiments: with and without insecticide (the same as in the field experiment). To examine whether the insecticide inhibited seed germination, seeds of each tree species were sown in germination trays and covered by soil. Ninety seeds were sown per tray per treatment (two trays per species). Each treatment was placed far away from each other, to avoid possible insecticide drift on to the control. Germinating seeds were counted weekly and the percent germinant was compared between the two treatments (Figure 3.8).

Figure 3.8 Germination tray with seeds (1 module per seed) in nursery experiment.

3.3.2 Survey of potential seed predators

To identify potential predators, I collected the data on small mammals, birds and insects visiting the experimental plots.

Vertebrate species - small mammals and birds

Small mammals and birds were surveyed, using five camera traps for seven months from August 2015 to February 2016. The occurrence of vertebrate species was captured using RECONYX™ PC900 HyperFire™ cameras (set for five snapshots per detection). Each camera trap was mounted in a plastic case and attached to an iron bar, 40 cm above the ground (Figure 3.9). The camera traps were randomly placed across the site (Figure 3.1) - over seven months, a total of 28 trapping locations.

Small mammals and birds, detected by the camera traps, were identified to species or genus. We used "A Naturalist's Guide to the Mammals of Thailand and Southeast Asia" (Shepherd and Shepherd, 2012) and "Guide to the Birds of Thailand by Boonsong Lekhakul" (Nabhitabhata *et al*., 2012) for species identification. In addition, the time and date, the number of photos and the number of individuals were recorded and counted from the photographs; whenever a single species appeared in photographs taken more than 30 minutes apart, the two subjects were treated as separate individuals (O'Brien et al., 2003).

Invertebrate species - insects

To collect insect specimens, pit-fall traps, sticky traps and direct collecting were used. Insect collections were done three times, (1) in August (after sowing, but before most seeds had germinated), (2) in October (after the small-seedling stage at the end of the rainy season), and (3) in April (with larger seedlings in dry season).

Pitfall traps were randomly installed near seed or seedling stations. Fifteen plastic cups, filled with a mixture of water and liquid detergent (100 ml water: 1 ml detergent), were randomly installed at 5 points per replicate (5 x 3 replicates) for three days (Figure 3.10A). The cups were placed in a small hole on the ground, so that the cup opening was at the same level of the soil surface. A piece of small plastic sheet (size: 5 x 5 cm) was placed to prevent additional accumulation of rain water, over of each pit-fall trap.

Fifteen sticky traps (size: 15 x 15 cm^2), made from yellow corrugated plastic with glue for insect trapping, were installed next to pitfall traps 10 cm above the ground (using 20 cm bamboo sticks as poles) (Figure 3.10B). Moreover, at the time of censusing seed removal and germination, insects that were found in the experimental plots were collected by hand.

Insect specimens were preserved in 75% alcohol and classified to Order and Family, according to their morphological characteristics using "An Introduction to the Study of Insects, 6th Edition" (Borror *et al.*, 1989).

Figure 3.9 Camera trap installed at the field experimental site

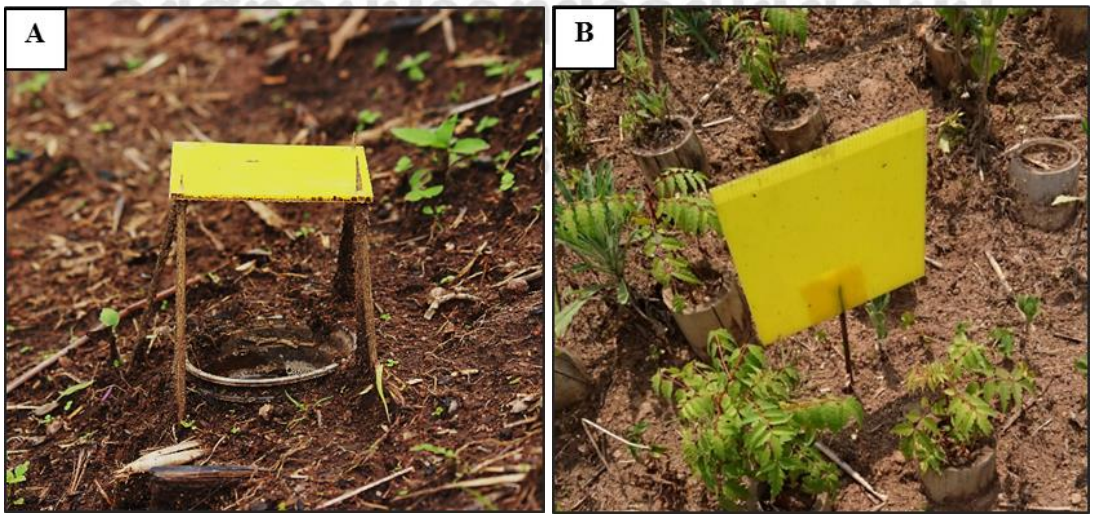

Figure 3.10 Pit-fall trap (A) and the sticky trap (B) in the study site.

3.4 Data collection and analysis

3.4.1 Seed removal and germination

The numbers of seeds removed from the bamboo tubes were counted weekly. Traces of seed removal were recorded, including scratches, digging marks and nests of some insects nearby the bamboo tubes. Germinated seeds were recorded as the presence of radicles and/or hypocotyl.

A generalized linear model (GLM) with a binomial family test in the R program ver. 3.4.1 was used to determine the effects of treatments on seed removal and germination. Species and treatments were used as independent variables. The dependent variable was the number of seeds removed or germinated.

Formula of GLM equation;

$$\mathbf{g(P) = \ln\left(\frac{P}{P-1}\right) + \beta_0 + \beta_1 * Species + \beta_2 * Treatment + error}$$

In addition to seed germination in nursery, survival analysis and log-rank test (Chi-square test, *df* = 1, Critical value = 3.84) was used to determine the difference between control and insecticide treatments. Furthermore, seed germination in the field and the nursery were also compared by survival analysis and log-rank test (Chi-square test, df = 1, Critical value = 3.84).

3.4.2 Cotyledon-seedling and leafy-seedling mortality

In this study, seedling mortality was used as an index to estimate seedling predation. Germinants were classified as cotyledonous-seedlings if they possessed expanded cotyledons with no true leaves. Later, they were classified as leafy-seedlings once at least the first pair of true leaves had fully expanded (Figure 3.11). The numbers of cotyledonous-seedlings and leafy-seedlings that were dead were recorded. In addition, physical signs of damage to the dead seedlings that remained on-site were recorded and classified into two categories: damage by insects and wilting. Dead seedlings that had disappeared from the site were classified as “unknown cause of death”.

Percent mortality of cotyledonous-seedlings and leafy-seedlings were dependent variables and analyzed separately. The effect of the treatments on cotyledonous- and leafy-seedlings mortality were analyzed by a generalized linear model (GLM) with a binomial family test using the R program ver. 3.4.1. Species and treatments were used as independent variables. The dependent variables were the numbers of dead and surviving seedlings. For each species the percent mortality per day of cotyledonous-seedlings and leafy-seedlings in the control treatment (control and open cage) were compared by t-test (two-tailed at significant level of 0.05).

Figure 3.11 Young seedlings of *P. cerasoides* (A) and *A. kurzii* (B) with cotyledons, but no true leaf. Small seedlings with both cotyledons and true leaves of *H. dulcis* (C) and *C. axillaris* (D)

Dead seedlings were classified into three categories according to their appearance:-

1) seedlings with only stem (no leaves present)

2) dry seedlings and

3) nothing observed (no seedling or stem in the bamboo tube).

3.4.3 Seedling survival and relative growth rates

Seedling growth parameters were monitored three times, in October 2015, April 2016 and after the dry season in July 2016 (Table 3.3). Measurements included seedling height, crown width, root collar diameter and health.

For seedling survival, the numbers of seedlings that survived after the dry season in July 2016 were recorded and percent survival of those that had germinated was calculated. A generalized linear model (GLM) with a binomial family test was carried out using the R program ver. 3.4.1 to compare differences among species. Tree species was used as the independent variable. The dependent variable was of mortality.

For seedling growth, the relative growth rate (RGR) of each species was calculated from changes in height (H), root collar diameter (RCD) and crown width (CW) of each surviving tree. The formula followed that of FORRU (2006): -

$$\mathbf{RGR} = \frac{\mathbf{\ln(final) - \ln(initial) \times 36{,}500}}{\mathbf{No.\ days\ between\ measurements}}$$

3.4.4 Seedling performance index (SI)

A species performance index (SI) was calculated using plant species traits from the field experiment, a year after sowing seeds (Table 3.2).

Table 3.2 Species performance index (SI)***** by seed removal and seedling traits

Features	Categories	Score	Rating score
Seed removal	less than 10%	4	Excellent
	10-35%	3	Acceptable
	34.9-50%	2	Marginal
	more than 50%	1	Unacceptable
MLD**	less than 30 days	4	Excellent
	30-50 days	3	Acceptable
	51-70 day	2	Marginal
	more than 71 days	1	Unacceptable
Germination	more than 70%	4	Excellent
	50-70%	3	Acceptable
	40-49.5%	2	Marginal
	less than 40%	1	Unacceptable
Seedling mortality (% from total germination)	less than 15%	4	Excellent
	14.9-30%	3	Acceptable
	29.9-50%	2	Marginal
	more than 50%	1	Unacceptable
Survival	more than 70%	4	Excellent
	50-69.9%	3	Acceptable
	40-49.9%	2	Marginal
	Less than 40%	1	Unacceptable
Seedling height	more than 50 cm	4	Excellent
	35.0-50.0 cm	3	Acceptable
	20.0-34.9 cm	2	Marginal
	less than 20 cm	1	Unacceptable
Crown width	more than 50 cm	4	Excellent
	35.0-50 cm	3	Acceptable
	20.0-34.9 cm	2	Marginal
	less than 20 cm	1	Unacceptable
RCD	more than 4.50 mm	4	Excellent
	3.25-4.49 mm	3	Acceptable
	2.00-3.24 mm	2	Marginal
	less than 2.00 mm	1	Unacceptable
RGR (% per year)***	more than 100%	4	Excellent
	75 -99 %	3	Acceptable
	50 - 74%	2	Marginal
	less than 50%	1	Unacceptable
Species rating (from total score above)	30 or more than		Excellent
	21 to 29		Good
	12 to 20		Marginal
	11 or less than		Poor

*The index modified from Elliott *et al*., 2003; Lu *et al*., 2016 and Lu *et al*., 2017)

**MLD: median length of dormancy time from sowing to final germination.

***RGR averaged from 3 parts: height, crown width and RCD

3.4.4 Potential seed predators

The photographs from the camera traps were used to calculated an index of species abundance, richness and distribution, based on the assumption that when the population density of animals increases, the possibility of capture by camera traps also increases (Abi-Said and Amr, 2011; Rovero and Marshall, 2009). We used the number of independent photographs to calculate the number per total effort of 100 trap days.

The numbers of individuals, Orders and Families of invertebrate species from pit-fall traps, sticky traps and direct handing were counted. In addition, insects were grouped according to their mouth parts characteristics 1) chewing, 2) sucking and 3) lapping, to indicate their diets.

The abundance of invertebrates (species diversity and evenness) was calculated and determined by Shannon's method (log base e) for each collection period (Shannon and Weaver, 1949). In addition, Sørensen similarity index was used to determine the similarity coefficient of invertebrate communities among three seasons (Diserud and Odegaard, 2007).

5.1 3.4.5 Variation of animal visits and seed-seedling transitional stage

Seed and seedling stage were recorded from the observation. The data were used to create a timeline of seed-to-seedling transition stages. Therefore, relevance between seed-seedling and predator were considered by timeline graph.