CHAPTER 3

MATERIALS AND METHODS

3.1 Study area and period

The experiments were conducted in two locations in northern Thailand representing both upland and lowland sites. Chiang Mai University (Muang District) and Partners Relief and Development research farm (Sansai District) were chosen to be representative of lowland sites in the northern part of the country. The Upland Holistic Development Project (UHDP- Mae Ai District) was chosen to be representative of upland sites in the northern part of the country (Fig. 4). Greenhouse and field trials were carried out in both sites.

Figure 4: Map of Thailand (Tourizm Maps, 2003)

Chiang Mai (18º47'N, 98º59'E) and Mae Ai (19º58'N, 99º19'E) are quite similar in climatic conditions. They fall under the category of tropical monsoon climate, getting an average yearly rainfall of about 1,249 millimeters per year, 89% of it falling between May and October. The average relative humidity is around 76%, but ranges between 60% in the dry season (November to May) to 85% in the wet

season (May to October). Temperatures average around 26 ºC, but range from 11 ºC in the cold season (November through February) to 37 ºC in the hot season (March through May). Because Chiang Mai is at a lower latitude and elevation (313 meters), rainfall and temperatures tend to be higher than in Mae Ai, which is situated at 487 meters elevation (Fig. 5).

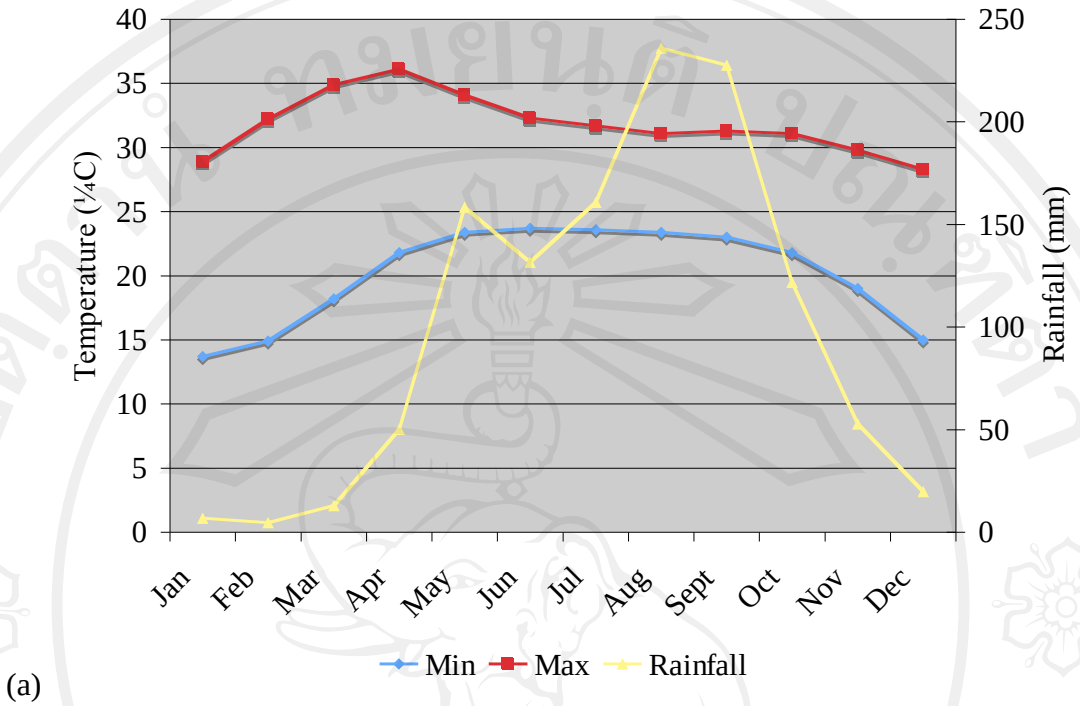
3.2 Experimental design

3.2.1 Greenhouse experiment

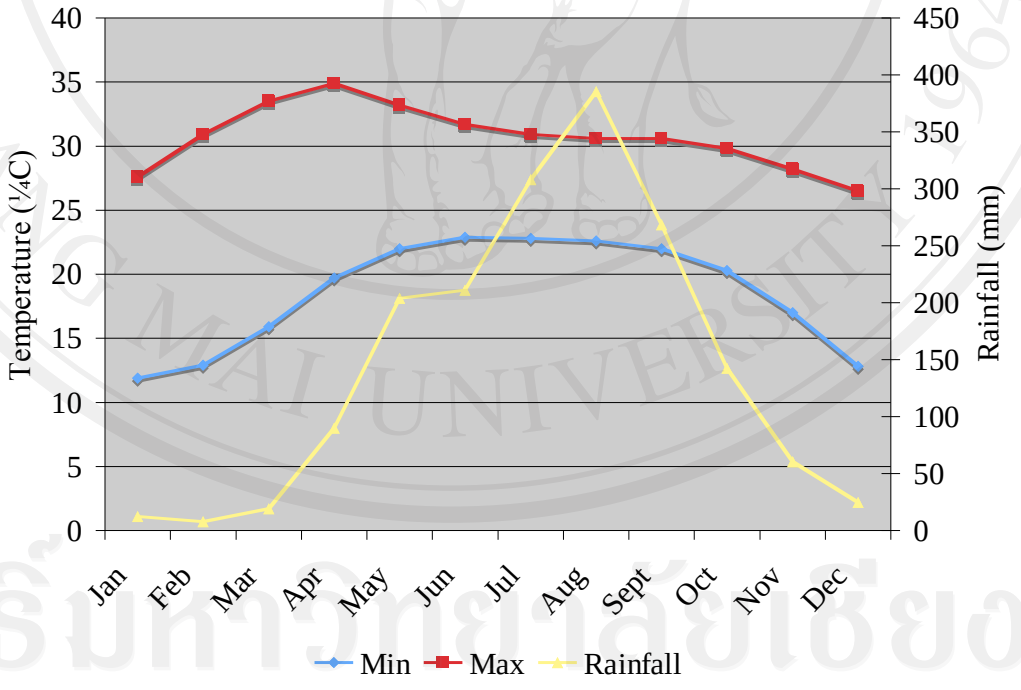
The greenhouse experiment was conducted as a Randomized Complete Block Design (RCBD) with eight replications at each of two sites, repeated twice at each site. Due to environmental gradients in the greenhouse, such as shading and distance to the edge of the bed, RCBD was the best option for experimental design. Replications were distributed across environmental gradients, within which the six different treatments were randomized. The experiment was carried out in both lowland and upland areas and done in both the dry season as well as the rainy season, running for two months each.

The six treatments were as follows:

- Control (GH1-Cont): The corm's cortex, including the roots, was peeled away and the pseudostem removed just above the transition zone (Fig. 6ab). Corms were dried overnight before being placed in a moist rice husk bed.



(a)



(b)

Figure 5: Average Temperature and Rainfall for (a) Chiang Mai and (b) Mae Ai (data from Chiang Rai) over a 30 year period (TMD, 2006b).

- Plants produced from stem fragments technique (GH2-PIF): The corm's cortex, including the roots, were peeled away, and the pseudostem cut off one sheath at a time, along the transition zone until the edges of the leaf sheath overlapped. The central apex of the pseudostem was cut about 2 cm above the last cut. After allowing corms to dry two days, the central apex was cut back little by little, until the tip of the apical meristem could be clearly discerned. The meristem was then damaged with three crosswise cuts through the central point and allowed to dry for several hours before planting (Fig. 6c).

- Dipping corm in BA (GH3-BA1): The corms were dipped (Fig. 6d) into 10^{-2} M BA for 30 minutes, and apical meristems were bored out (Fig. 6e). The corms were then allowed to dry overnight before being placed in a moist rice husk bed. In preliminary studies, 4 mL of BA was injected into the base of split-corm derived suckers (Osei, 2005), but because of the difficulty of injecting liquid into the banana stem base dipping was used instead.

- Dipping corm in BA half concentration (GH4-BA2): The corms were treated identically to those treated with BA but half the concentration was used ($5x10^{-3}$ M) (Fig. 6d).

- Dipping corm in coconut water (GH5-CW): The corms were dipped (Fig. 6d) into strained coconut water from mature green coconuts, obtained at a local Chaing Mai market, for 30 minutes and apical meristems were bored out (Fig. 6e). The corms were then allowed to dry overnight before being placed in a

moist rice husk bed. In preliminary studies, 6 or 8 ml of coconut water was injected into the base of split-corm derived suckers (Osei, 2005), but because of the difficulty of injecting liquid into the banana stem base dipping was used instead.

- Split corm (GH6-SC): The pseudostems and roots were removed, leaving only the peeled corm. This was cut into quarters (Fig. 6f), and dried overnight before placing in a rice husk bed (Dzomeku, 2000).

3.2.2 Field experiment

The field experiment was conducted as a Randomized Complete Block Design (RCBD) with five replications at each of two sites. Due to environmental gradients in the field, RCBD was the best option for experimental design. The field was divided into blocks across environmental gradients, within which the five treatments were randomized. The experiment was carried out in both lowland and upland areas and done in the rainy season, running for four months.

Field blocks were selected according to environmental gradients, taking into account slope, relation to sun, soil type, and location on the farm. There was no irrigation, as low-income farmers typically do not irrigate their bananas. Prior to conducting the experiment, covariate measurements were taken of the mother plants' height (from soil level to top of pseudostem – where base of newest leaf emerges) and pseudostem circumference (at soil level), along with measurements of the suckers' height from soil level and pseudostem circumference. Number of suckers present at the beginning of the experiment was counted.

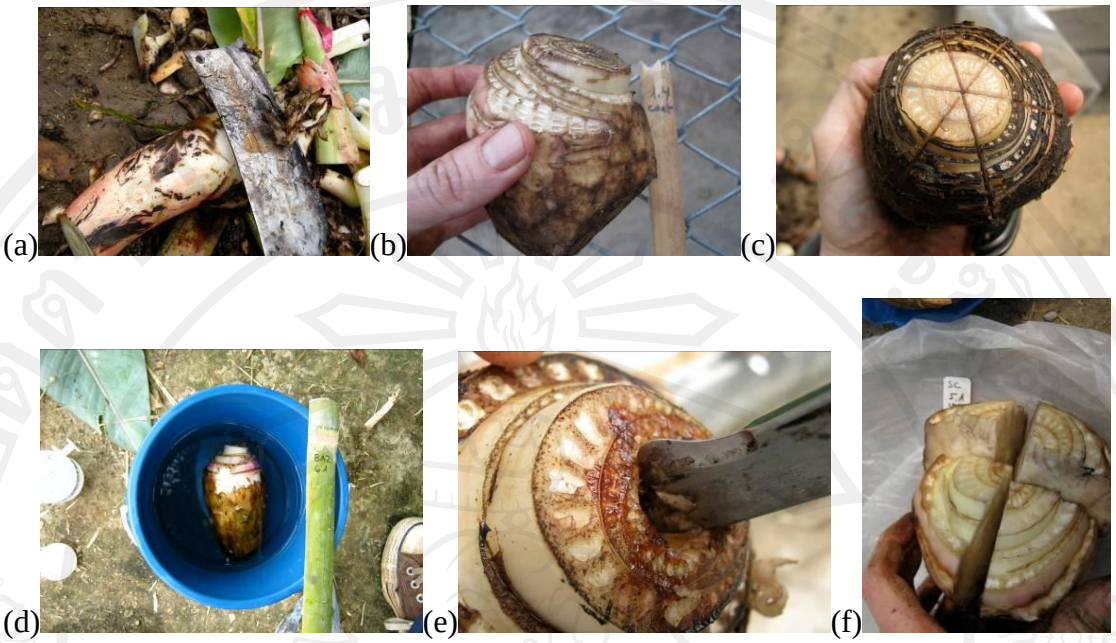


Figure 6: Treatment of greenhouse corms: (a) peeling cortex, (b) control corm, (c) PIF treatment, (d) dipping in cytokinin solution, (e) digging out apical meristem, and (f) split-corm treatment.

The five treatments were as follows:

- Control (F1-Cont): Banana mats were observed under natural growing conditions, without any manipulation to the plant.

- Staking the mother plant (F2-MM): Young mother plants were bent over at the top, at a height of about 2 m, (Fig. 7a) then a thin, flat stake, 5cm wide, was driven into the center of the pseudostem at a height of about 15 cm (Fig. 7b).

- BA pooled in sword sucker (F3-BA1): All suckers under one meter were exposed at the base of the mother plant. The pseudostems were removed 2 cm above the rhizome collar and the apical meristem bored out. The remaining

fragment was then cut with crosswise incisions, cutting down to the transition zone, and the hole filled with 4 mL BA (10^{-2} M concentration) (Fig. 7c). The depressions were then covered with enough of a mixture of soil, rice husks and manure to cover the corms completely, before filling the rest of the depressions in with top soil.

- BA half concentration pooled in sword sucker (F4-BA2): The corms were treated identically to those treated above but half the concentration of BA was used ($5x10^{-3}$ M).

(a)  (b)  (c) 

Figure 7: Treatment of field bananas: (a) bending top of mother plant with stake, (b) staking mother plant, (c) cytokinin pooled in corm.

Table 1: Greenhouse and Field Experiment Treatments across Sites

	GREENHOUSE	FIELD
LOWLANDS (Chiang Mai)	GH1-Cont GH2-PIF GH3-BA1 GH4-BA2 GH5-CW GH6-SC	F1-Cont F2-MM F3-BA1 F4-BA2 F5-CW
UPLANDS (Mae Ai)	GH1-Cont GH2-PIF GH3-BA1 GH4-BA2 GH5-CW GH6-SC	F1-Cont F2-MM F3-BA1 F4-BA2 F5-CW

Treatment: control (Cont), plants produced from stem fragments (PIF), benzylaminopurine 10^{-2} M (BA1), coconut water (CW), split-corm (SC), and benzylaminopurine $5x10^{-3}$ M (BA2). Experiments: greenhouse (GH), field (F).

- Coconut Water Pooled in Sword Sucker (F5-CW): All suckers less than one meter were exposed at the base of the mother plant. The pseudostems were removed about 2 cm above the rhizome collar and the apical meristem bored out. The remaining fragment was then cut with crosswise incisions, cutting down to the transition zone, and the hole filled with 4 cc coconut water (Fig. 7c). The rhizomes were then covered with a mixture of soil, rice husks and manure.

3.3 Materials preparation

3.3.1 Plant materials

3.3.1.1 Greenhouse corm preparation

Musa (ABB) cv. 'Kluai nam wa' sword suckers (<100 cm height) were used for greenhouse trials, and were collected on the UHDP uplands site and from a local farmer in the Sansai district for the CMU lowlands site. In all greenhouse trials, sword suckers, less than one meter tall, were dug five days before re-planting. Replications were laid out to have similar sized corms distributed throughout blocks and randomly assigned to treatments.

The following sequence was used for greenhouse corm preparation:

Day 1 – Banana suckers were dug and measurements were collected from mother plants (height and circumference) and suckers (height, circumference and weight).

Day 2 – PIF treated corms were prepared in order to allow them the two days of drying described in literature.

Day 3 – Greenhouse bed was treated with fungicide.

Day 4 – Remaining corms (Cont., BA1, CW, SC, and BA2) were treated.

Day 5 – Corms were planted in greenhouse bed.

For all corms, the corm cortex was peeled away with a knife in order to cut away any potential pest, nematode or disease damage present and the pseudostem was removed, one leaf sheath at a time, just above the transition zone until edges of the leaf sheaths fully overlapped. Leaf sheaths were removed individually in order to keep from damaging the apical meristem. Ethyl alcohol was used to clean knives between every corm.

As a side note, after applying fungicide to the rice husks during the first repeat of the UHDP greenhouse trial, the temperature of the bed began increasing (most likely due to the beginning stages of decomposition). Measured temperatures reached above 50 ºC. In order to keep the corms from being killed, and because they had already been treated by the time it was discovered, corms were left out overnight to see if the temperature would decrease. When temperatures continued to rise, corms were placed in refrigeration for two nights while the rice husks were watered two times per day in order to lower the temperatures. After planting, temperatures were still slightly raised, but beds continued to be watered twice a day until the temperature was regulated. In order to keep consistency between trials, the corms in the second UHDP trial were also put in refrigeration for two nights before planting although the rice husk bed did not exhibit elevated temperatures.

3.3.1.2 Field banana suckers preparation

Musa (ABB) cv. 'Kluai nam wa' sword suckers (<100 cm) were also used for field trials. In both field trials, sword suckers, less than one meter in height, were used. Because of the way the farms were set up, banana plants were not all planted at the same time and it was difficult to find 40 mats of the same age. Thus, each block was chosen to have banana mats close to the same age and in similar environmental gradients. For the treatments of Cont, BA1, BA2, and CW, plant mats were selected with no more than three mother stalks and no more than seven suckers. In contrast, for the MM treatment, younger mats were selected, with only one or two mother stalks and no more than one sucker. Replication groups were selected then treatments were randomized and assigned within each block.

The following sequence was used for field sucker preparation:

Day 1 – Soil around banana suckers was dug in order to fully expose the base of all the corms for BA1, BA2 and CW treatments. Height and circumference measurements were collected from mother plants and suckers.

Day 2 – Treatments were applied.

Ethyl alcohol was used to clean knives between each sucker.

3.3.2 Cytokinin preparation

6-Benzylaminopurine (Fig. 8a) was obtained from Sigma-Aldrich GmbH. It was prepared as a 10^{-2} M concentration as used in experiments done by Osei (2005). An additional half concentration ($5x10^{-3}$ M) was used to determine if there was a significant difference in numbers of plantlets produced. Samples were prepared at the Central Laboratory, Faculty of Agriculture, Chiang Mai University, Chaing Mai, Thailand.

For the 10^{-2} M concentration, accurately weighed 2.25 g of BA were dissolved in a 1 M solution of NaOH (4 g NaOH dissolved in 100 mL distilled water). NaOH was added 1 mL at a time, using 40 mL, until BA was dissolved. This was then diluted to 1 L in a volumetric flask with distilled water. The half-concentration used the same procedure, but 1.126 g of BA were used instead, dissolved with 1 M NaOH solution, using 20 mL of 1 M NaOH. This was then diluted to 1 L in a volumetric flask with distilled water.

Fully mature, green coconuts (Fig. 8b) were bought from a local market in Chiang Mai. Three coconuts were bought for each trial, mixing the coconut water contents together for use, and setting aside enough for the field trails. Three coconuts produced about 1.5 L coconut water. Coconuts were not opened until just before use to keep the water fresh as it ferments rather quickly after being removed from the coconut. The amount set aside for field trials was placed in a refrigerator until usage.

(a) 

(b) 

Figure 8: Preparation of cytokinins included (a) 6-Benzylaminopurine and (b) mature, green coconuts.

3.3.3 Greenhouse planting bed setup

Planting beds were prepared at CMU and UHDP (Fig. 9) 1-2 weeks prior to corm treatment. They measured about 2 m x 75 cm x 40 cm. Due to its wide availability in developing world tropics, rice husks were selected as the growing media. The rice husks were purchased locally and treated with fungicide, pentachloronitrobenzene, by diluting 60 mL in 20 L of water and evenly pouring the solution over the bed two days prior to planting.

The treated corms were positioned randomly, 3-6 cm apart (Fig. 9c), in eight replicated blocks at each site. Different sized corms were placed randomly in each replication in order to make all replications have consistent sized corms. The beds were covered with clear plastic to simulate greenhouse conditions and covered with 50% shade cloth to protect the new leaves from sunburn. Watering occurred weekly, supplying enough water to fully saturate the rice husks. This kept the bed moist throughout the week, as rice husks have a high water holding capacity. Each experiment at each site was repeated for two months in the rainy season (11.09.11-14.11.11 in the uplands and 21.09.11-24.11.11 in the lowlands) and two months in the dry season (07.12.11-08.02.12 in the uplands and 30.11.11-30.01.12 in the lowlands). These repeats were a proxy for the rainy and dry seasons as the experiments actually started late in the seasons.

(a)  (b)  (c) 

Figure 9: Greenhouse planting beds at (a) CMU and (b) UHDP. Corms were positioned in randomized complete blocks (c).

3.4 Data collection

Before treatments were applied, measurements were taken from banana mats in both greenhouse and field experiments, including:

1. Number of mother stalks,
2. Height and circumference of mother stalks,
3. Height and circumference of sword suckers and
4. Weight of corms dug (greenhouse trial only).

After treatment, dependent variable data collection took place on a weekly basis, and included:

1. Date of plantlet emergence,
2. Number of emerged plantlets per corm and
3. Height and circumference of plantlet and sucker pseudostems.

Height was measured from the transition zone (where outer leaf sheath meets corm) to the top of the pseudostem, the base of the youngest leaf. Circumference was measured at the soil or greenhouse substrate surface. Emergence was measured as the date the plant pushed through soil/substrate surface. The number of plantlets was evaluated as the number to survive until the end of 60 days for the greenhouse experiments and 120 days for the field experiments.

3.5 Data Analysis

3.5.1 Temperature Analysis

To determine greenhouse temperatures, the HOBO® Data Logger (1996) was used. It was positioned under the plastic of the greenhouse bed, hanging above the substrate. Temperature readings were taken every two hours. From these

readings, daily averages, daily maximum and minimum temperatures were used to display temperature charts along with the base temperature for banana (14 ºC). Ambient temperature was obtained from local weather stations in Chiang Mai and Chiang Rai (Wunderground, 2012ab).

In order to look at the role of temperature in the emergence of plantlets, growing degree days (GDD) were used. GDD are a measure of accumulated heat. The formula, GDD = $\Sigma[T_{avg} - T_{base}]$ was used, where T_{avg} used weekly temperature averages from data loggers placed in greenhouses and T_{base} = 14 ºC (Robinson, 2010), as the base temperature for bananas, below which leaf growth stops.

Presenting the number of emerged field plantlets is a little misleading because different numbers of mother plants were present for different banana mats, as well as different numbers of treated suckers. Unlike the greenhouse experiment, it is difficult to analyze which treatments produced the most plantlets. It was not possible to tell at the end of the experiment which plantlets actually were produced from treated suckers, so the ratio of emerged plantlets was used as a more precise proxy for the number of emerged plantlets. This was done using a ratio of the number of emerged plantlets to the number of treated suckers, or, in the case of MM, number of mother stalks present, since many of the replications had no initial suckers.

3.5.2 Statistical Analysis

Data for the greenhouse and field experiments were analyzed separately as linear mixed models using the MIXED Procedure of SAS[1]. Site, repeats (greenhouse only- analyzed as a fixed effect to parse out its potential for influencing treatments based upon clear climatic fluctuations over the experimental period), and treatments were analyzed as fixed factors and block was a random factor, and was nested in repeats and site. For all dependent variables, degrees of freedom were adjusted using the Satterthwaite correction (Littell et al. 2002), and normality of the raw data and residuals was evaluated using the UNIVARIATE procedure of SAS. When factors were significant, means were separated with Fisher's Protected LSD Test at an alpha = 0.05 using the PDMIX800 macro (Saxton 1998).

When running the statistical analysis for the greenhouse experiment, the control treatment was removed from dependent variables of time to emergence and mean final circumference. The control emerged much earlier, with a larger circumference than any other plantlets, skewing the best-fit curve. Technically, the plant produced from the control treatment was not a plantlet, deriving from lateral buds, thus should not be considered in comparison of the other treatments.

[1] SAS statistical software, SAS Institute Inc., 100 SAS Campus Drive, Cary, NC 27513