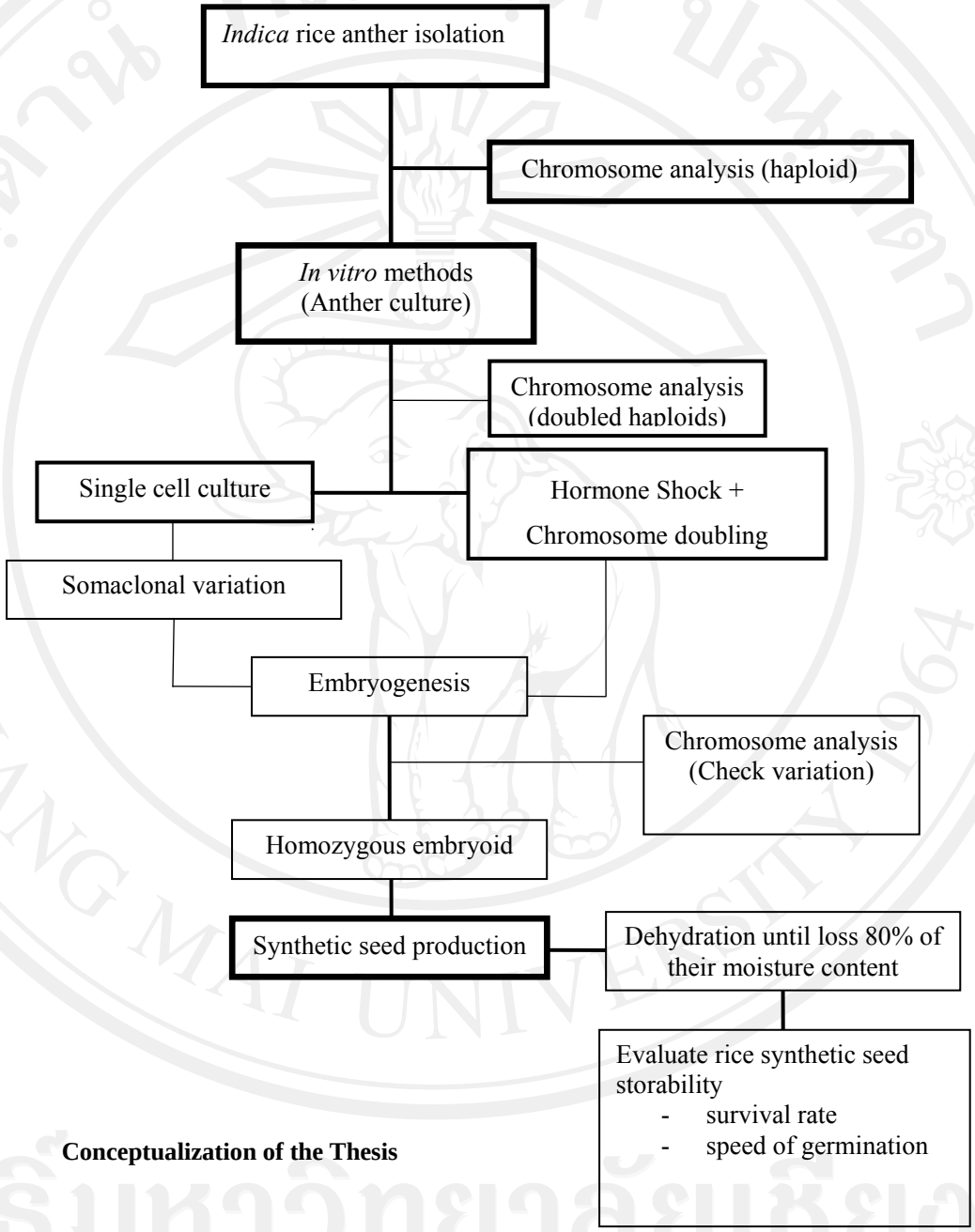
Chapter 3

Materials and methods

Scopes and Locations of the Study

The study consisted of 3 parts. The first part is the chromosome analysis. The second part is the study of *in vitro* culture and chromosome doubling. The third part is the study of the role of embryoids synthetic seed production. All parts are done at Plant tissue culture laboratory, the Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office, Office of Higher Education Commission, Ministry of Education. (AG-BIO/PERDO-CHE). Faculty of Agriculture, Chiang Mai University.



Conceptualization of the Thesis

3.1 Plant materials :

Donor plants (KDML 105 x SPR 1 seeds) were grown in field and *in vitro* to produce F1. Seeds of *Oryza sativa* L. subspecies *indica* were obtained and planted. Plants are usually ready for anther culture about 60 to 90 days after planting. The KDML 105 x SPR 1 *(indica x indica*) anthers were used for the study. Stems containing panicles with pollen at this stage can be identified in rice by the relative positions of the flag leaf and penultimate leaf collars. Anthers from the distance 4–9 cm. between the base of the flag and auricle of the last leaf which were in the middle to late uninucleate stage of development before pollen mitosis were collected.

3.2 Chemical reagents :

3.2.1 *In vitro* culture media

- Macronutrients

NH_4NO_3

KNO_3

$MgSO_4 \cdot 7H_2O$

KH_2PO_4

$CaCl_2 \cdot 2H_2O$

- Micronutrients

H_3BO_3

KI

$MnSO_4 \cdot 4H_2O$

$ZnSO_4 \cdot 7H_2O$

$Na_2MoO_4 \cdot 2H_2O$

$CuSO_4 \cdot 5H_2O$

$CoCl_2 \cdot 6H_2O$

Na_2-EDTA

$FeSO_4 \cdot 7H_2O$

- **Amino acid/Vitamins/Sugar**

Glycine

Nicotinic acid

Pyridoxine HCl

Thiamine HCl

Myo-inositol

Sucrose

- **Plant growth regulator**

Naphthaleneacetic acid (NAA)

2,4- Dichlorophenoxyacetic acid (2,4-D)

6- furfurylaminopurine or Kinetin (Kn)

3.2.2 Haploid diploidization (Chromosome doubling)

- N-[(7S)-1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl] acetamide (Colchicine)

3.2.3 Chromosome analysis

- ethanol
- acetic acid
- 1 N HCl
- aceto-orcein

3.2.4 Synthetic seed production

- LS liquid media
- benomyl
- sodium alginate
- calcium chloride solution
- distilled water

All chemicals are available as preweighed packets from Sigma-Aldrich Co. LLC.

3.3 Instrument

- Laminar flow hoods
- Autoclaves
- Distillation unit
- Orbital shaker
- Stereo microscope

- Compound microscope
- Cold storage (refrigerator)

3.4 Equipments :

- Sterilization equipment
- Balance(s)
- Water purification
- pH meter
- Magnetic stirrer
- Micropipettor(s)
- Cell counter
- Culture containers
- Pipettes
- Pasteur pipettes
- Sterilization filters
- Pipette tips
- Tissue culture flasks and bottles
- Petri dishes
- Forceps
- Scalpel

3.5 Methods :

3.5.1 Pollen culture :

The F1 hybrid panicles from KDML 105 x SPR 1 *(indica x indica*) were collected between 9.00 to 10.00 a.m. on sunny days when the distance between flag leaf and penultimate leaf was 5-9 cm. Anthers of mid to late uninucleate stage observed by microscope. This experiment was done in 3 replicates with 300 cells per treatment. (microscopic observation) were obtained from the spikelets of middle part of the panicles. The panicles were wrapped in aluminium foil with moist cotton at the base of cut panicle surface and stored in the dark for 7 and 14 days at 5, 10 and 15ºC in sealed polyethylene bags. The panicles were rinsed with 70% (v/v) ethanol for 20 seconds. The spikelets were removed and they were surface sterilized with 10% (v/v) sodium hypochloride solution for 20 minutes and rinsed thoroughly with sterilized distilled water 3 times. Individual florets were cut and separate from the spikelets after that separate the anthers out of each floret using a pair of forceps with bend ends. All six anthers from each floret were removed without any damage. Anthers were removed from the spikelets and cultured 10 anthers 5x8 cm steriled glass bottle with LS solidified medium.

3.5.2 Caulogenesis inducement:

Ten formulas of LS media were used: (1) 2 mg/L of 2,4-D (control); (2) 2 mg/L of 2,4-D + 2 mg/L of naphthalene acetic acid (NAA); (3) 2 mg/L of 2,4-D + 2 mg/L of kinetin; (4) 2 mg/L of 2,4-D + 2 mg/L of kinetin + 2 mg/L of NAA; (5) 10 μM KNO_3 + 2 mg/L of 2,4-D + 2 mg/L of NAA; (6) 10 Mm NH_4NO_3 + 2 mg/L of 2,4-D + 2 mg/L of NAA; (7) 2 mg/L of 2,4-D + 2 mg/L of NAA + 15% coconut water; (8) 2 mg/L of 2,4-D + 2 mg/L of NAA + 20% coconut water; (9) 10 μM KNO_3 + 2 mg/L of 2,4-D + 2 mg/L of NAA + 20% coconut water; and (10) 10 μM KNO_3 + 2 mg/L of 2,4-D + 2 mg/L of NAA + 20% coconut water + 1 mg/L of activated charcoal). Macronutrient concentrations (KNO_3, NH_4NO_3), growth regulators (2,4-D, NAA) and other organic compounds (coconut water and activated charcoal) were modified and applied in order for embryogenic callus to be transformed to the embryo-like structure (ELS). The pH of each composition was adjusted to 5.8 before autoclaving at 115◦C for 15 min. The anthers were cultured in a 5×8 cm sterile glass bottle, 10 anthers were inoculated and 3 replicas (100 anthers per replica) were cultured in each treatment which contained 25 mL callus-inducing medium. The cultures were kept at 25 ± 2◦C under continual illumination from white fluorescent lamps (3,000 Lux under 16 h photoperiods). Callus was formed during 4–6 weeks. After producing doubled haploid plantlet from embryogenesis in next experiment, the same procedure was used again with doubled haploid anther (H1 anther) to compare the responses to anther culture process for callus and plantlets.

3.5.3 Hormone Shock, Doubling chromosome and Embryogenesis inducement:

LS media supplemented with various concentrations of colchicines and 2,4-D (Tables 1 and 2) were used for embryoids inducement. The 2 mg calli was transferred to LS liquid media in which 2 mg/L NAA, 1 mg/L kinetin, 1 g/L sodium salt of 2-(N-morpholino)-ethanesulfonic acid, 1 g/L casein hydrolyzate, 30 g/L sucrose, 30 g/L sorbitol and 0, 0.1, 0.2, 0.3, 0.4 and 0.5 g/L colchicines were added. The comparison was done with hormone shock by culturing on LS media with various high concentrations of 2,4-D (50, 100 150 and 200 μM). The treatments took 12 hours and then those calli were subcultured to LS media without supplemented 2,4-D in a 250 mL Erlenmayer flask and placed on a rotary shaker at 100 rpm at 25±2 °C under continual illumination from white fluorescent lamps (3,000 Lux under 16 h photoperiods). Subculture of cell suspension into induced embryoid media formula was carried out for every 3 to 6 weeks in order to induce embryoids.

3.5.4 Chromosome analysis :

Analyzed metaphase chromosome of ELSs after treating with colchicines and 2,4-D by acetoorcein squash method was done according to Giri and Giri (2007). Anthers were fixed from 4 to 24 h in ethanol: acetic acid (3:1). After removing the embryoid from the fixative, it was hydrolyzed with 1 N HCl for 4 min at 60°C. Then HCl was removed and replaced with aceto-orcein at 90°C for 1–2 min. ELSs were squashed and observed ploidy by microscope. This experiment was done in 3 replicates with 100 cells per treatment. Compare somaclonal variation of rice chromosome after treating cochicine.

3.5.5 Synthetic seed production :

Add somatic embryos at the late proembryo stage cultured in modified LS liquid media which is the maturation formula containing 0, 0.2, 0.4 and 0.6 mg/l benomyl to autoclaved 3 % weight by volume sodium alginate. Use wide mouth 3 mm. diameter dropper to suck LS media containing somatic embryos then dropped into 75 mM calcium chloride solution. Stirring the solution for 30 minutes will produce complete synthetic seeds. Pour calcium chloride solution slightly off. Rinse synthetic seeds with distilled water which has been autoclaved for three times. Collect the beads by filtering through a nylon mesh. Wipe the seeds with sterized tissue papers. Finally, Select one somatic embryo synthetic seed out for the next experiment.

Pursue dehydration by using silica gel until synthetic seeds lost 80 % of their moisture contents. At this process, choose only seeds with single somatic embryos to test the survival rate and germination speed by culturing at 25±2 °C for 16 hours per day photoperiod for one week. Then record the survival rate and germination speed.

Store synthetic seeds in 250 ml. Erlenmayer flask, 20 seeds for each before sealing them with parafilm and store them at 25±2 °C in darkness for 2, 4, 6 and 8 weeks, respectively. After that, rehydrate those seeds, which have been stored in different period of storage, before testing the survival rate and germination speed by culturing them at 25±2 °C for 16 hours per day photoperiod for one week on MS basal medium for plantlet formation. Finally, record the result of test. Repeat this process for 3 times with 5 seeds for each.

Variables evaluated

After store synthetic seeds at 25±2 °C in darkness for 2, 4, 6 and 8 weeks, respectively, an evaluation was made of the percentage of encapsulated of somatic embryos and that presented germination and of the survival rate. Subsequently, at 2 weeks the percentage of germination of encapsulated somatic embryos was evaluated. The criterion used to evaluate the germination of the encapsulated embryos was the emergence of the root from the artificial capsule. The survival percentage of the embryoids was evaluated and every four days the percentage of germination reached was evaluated, taking the emergence of the root from the capsule as a criterion.

3.5.6 Statistical Analysis :

The caulogenesis and organogenesis percentages were shown according to LS media formulas. Kruskal Wallis test was used to determine caulogenesis and organogenesis frequency differentiation among various LS media formulas. Statistical analysis will be done by Analysis of Variance (ANOVA) and the means will be compared by Least Significant Difference (LSD).