ANDROGENESIS OF SELECTED INDICA RICE (*Oryza sativa* L.) GENOTYPES AND F_1 HYBRIDS

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The thesis attached here to, entitled ***"Androgenesis of Selected Indica Rice (Oryza sativa* L.*) Genotypes and F_1 Hybrids"*** was prepared under the direction of chairperson of the candidate's supervisory committee and has been approved by all members of that committee and the board of examiner as a partial fulfillment of the requirements for the degree of **Master of Agricultural Science (Horticulture and Agricultural Biotechnology)**.

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DECLARATION OF ORIGINALITY

This thesis represents the original work of the author, except where otherwise stated. It has not been submitted previously for a degree or to any other University.

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DEDICATED TO MY BELOVED PARENTS,
U NYUNT SAN AND DAW WIN SHWE

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ABSTRACT

This experiment was carried out to evaluate the callusing ability and green plant formation of selected indica rice genotypes and F_1 progenies after crossing selected genotypes. Shwe Man-1, Sin Akari-3, Sin Thwe Latt, Thee Htat Yin and Yar-8 genotypes were selected as parental lines based on callusing ability of anther culture. Five crosses (Shwe Man-1 x Thee Htat Yin, Thee Htat Yin x Sin Thwe Latt, Sin Thwe Latt x Sin Akari-3, Yar-8 x Thee Htat Yin and Sin Thwe Latt x Yar-8) were obtained by crossing the selected indica rice genotypes. Anthers of selected indica rice genotypes and F_1 hybrids were cultured on N_6 medium with supplemented 2 $mg \cdot L^{-1}$ 2,4-D and 0.5 $mg \cdot L^{-1}$ kinetin for callus induction. Calli were transferred onto MS medium with 1 $mg \cdot L^{-1}$ IAA, 1 $mg \cdot L^{-1}$ NAA, 1 $mg \cdot L^{-1}$ BAP and 2 $mg \cdot L^{-1}$ kinetin.

All tested genotypes showed callus formation except one cross (Sin Thwe Latt x Sin Akari-3) that showed no response on anther culture. Callus induction percentage of responsive genotypes varied from 0.1-2.6%. Green plant formation from the regenerated calli varied from 6.3-37.0%. Among parents, Yar-8 and Shwe Man-1 gave the highest callus induction and green plant formation. Cross involving Yar-8 as female parent, Yar-8 x Thee Htat Yin increased callusing and green plant formation ability. These three genotypes (Yar-8, Shwe Man-1 and cross of Yar-8 x Thee Htat Yin) produced green plants. The anther-derived plants of these three genotypes were haploid and double haploid. According to the results, Yar-8 genotype has the potential for double haploid production through anther culture in rice breeding program.

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CHAPTER I
INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereal crops cultivated in the world. It provides food for more than half of the world population (Sasaki, 2005). In particularly, nearly 90 % of the world's rice is produced in Asia. In Myanmar, rice is the most important dominating crop and is extensively grown throughout the country. Therefore, increasing rice production to support food security has been given a first priority in agricultural development in Myanmar. Indica rice varieties are mainly grown but some japonica rice varieties are rarely cultivated. The total rice growing area was 7.28 million hectares with an average yield of 3.84 MT/ha (MOAI, 2015). Country was benefited by earning foreign exchange by production and export of rice. However, there are some limitations for farmers to cultivate good quality rice. It needs to develop high yielding varieties with desired characters in rice production

Nowadays, there have been many problems faced with food shortage all over the world. Causes of decreasing food production are increasing global population, environmental degradation and climate change. Therefore, scientists are interested in these challenges and have been tried to upgrade production technologies. For rice growing countries, technologies in rice production must be promoted to supply the needs of population.

Plant breeders used conventional methods such as hybridization, selection, mutation, etc, to produce new rice varieties. The conventional plant breeding methods can be achieved by combining the desired traits through crossing with another desired characters. However, rice yields are affected by biotic and abiotic stresses. They attempt to improve rice by using biotechnology. Therefore, plant tissue culture has become an important tool for breeding improvement in rice (Ge *et al*., 2006). Various tissue culture techniques such as anther culture, protoplast fusion, leaf culture, root culture and dehusked seed culture are being applied for varietal development of cereal crops including rice in different countries (Dorosieve, 1996). Among these techniques, anther culture is the simplest and more efficient method (Niizeki and Oono, 1968).

The important role of anther culture in breeding program is to provide plants with special agronomic characters; development of earliness, increased grain weight, superior grain quality, pest and disease resistance (Zhang, 1984). Recently, anther

culture technique has been widely used in breeding program of many crops. It can be used as an effective and time saving for obtaining homozygous lines in varietal improvement (Chu, 2002). It has become a powerful tool for the rapid production of haploid and inbred lines used to obtain hybrid cultivars. Production of double haploids through anther culture is a rapid approach to homozygosity that shortens the time required for the development of new rice cultivars as compared to conventional methods, which require at least 6-7 generations. However, the usefulness of this technique is limited because some genotypes respond poorly to anther culture (Sopory and Munshi, 1996). Anther culture involves two steps; callus induction from microspores and green plants regeneration from calli. In the case of *indica* rice, major problems are early anther necrosis, poor callus proliferation and albino plant regeneration (Chen *et al*., 1991).

Callus induction and green plant regeneration is a pre-requisite for utilization of anther culture in breeding programs. Unfortunately, low percentages of both callus induction and plant regeneration are the principal constraints in successful anther culture in some rice varieties especially in *indica* rice since these critical culturing responses are genotype dependent (Roy and Mandal, 2005). The anther culture response depends on many factors such as various genotypes, physiological age of donor plant and culture medium (Torbert *et al.*, 1998). Breeders can overcome the barrier due to genotypes by crossing highly responsive to non responsive genotypes in rice breeding. To obtain good combinations with high callus induction and green plant regeneration, suitable parents could be selected for hybridization (He *et al.*, 2006).

Therefore, the objectives of this study were

(1) To evaluate the callusing ability of selected indica rice genotypes and F_1 progenies after crossing of selected genotypes

(2) To examine the green plant formation of selected indica rice genotypes and their F_1 hybrids

CHAPTER II
LITERATURE REVIEW

2.1 Anther Culture

Anther culture is a way of producing haploid plants ('n' chromosome number). The haploid productions in plant breeding using conventional method have been realized for long time. The conventional method requires at least 6-7 generations to get homozygous line. Hu and Zeng (1984) suggested that the double haploid technique can induce homozygous lines in fewer generations by doubling chromosome through inhibiting their anaphase.

Method of haploid production was rapidly expanded using tobacco (*Nicotiana tabacum*) which become the model species for anther culture experiments (Reed, 1966). This method has been applied to economic important crops such as potato and chilli (Wang *et al*., 1973), and wheat (Ouyang *et al*., 1973). Exploitation of anther culture technique is limited due to low regeneration frequency of anthers in rice, particularly in indica rice (Balachandran *et al*., 1999). Success of haploid induction depends on many factors; genotype, microspore developmental stage, cultivated conditions of plants, components of culture medium, panicle treatments (Shigh-Wei and Zhi-Hong, 1991; Balachandran *et al*., 1999; Wang *et al*., 2000; Datta, 2005 and Cha- *et al*., 2009).

2.2 Application of Anther Culture Technique to Rice Breeding

Since the first haploid plants were regenerated from rice anther in 1968 by Niizeki and Oono, anther culture technique has been integrated in rice breeding programs. A large number of rice varieties has been developed through anther culture and released for cultivation over several thousand hectares in China (Chen, 1986). Rice anther culture offers homozygous lines from heterozygous breeding lines. This method allows for selection efficiency due to better discrimination between genotypes within any generation and efficient retention of desirable alleles in later generation (Croughan and Chu, 1995).

The application of rice anther culture was become one of the alternative ways in breeding programs. This technique shortens the time required for the development of new varieties compared to conventional breeding methods (Gioi and Tuan, 2004). Many varieties of rice produced by anther culture technique and *in vitro* selection

have improved some characteristics such as pest resistance (Sun *et al*., 1992); disease resistance (Bumbang *et al*., 2010); grain quality (Thanh, 2011).

Plant breeders need to adapt speed and efficiency to develop new varieties, which is important for breeding program (Tuvesson *et al*., 2007). Breeders have produced many double haploid (DH) lines which have superior grain quality characteristics (Patil *et al*., 1997), resistance to blast, bacterial blight (Lee *et al*., 1989; Pauk *et al*., 2009), resistance to brown plant hopper and stem borer (Chung, 1987), resistance to salt tolerance and drought tolerance (Senadhira *et al*., 2002).

Recently, Thomson *et al*., (2010) developed DH lines from the crosses involving salt tolerant rice lines for saline areas in Bangladesh. Rice anther culture has been used to produce DH lines with multiple stress tolerances. Dewi *et al.,* (2009) reported that the anther culture ability of indica rice genotypes used for development of new rice varieties tolerant to aluminium toxicity. Similarly, Purwoko *et al*., (2010) also reported that DH lines tolerant to aluminium stress were produced through anther culture in upland rice genotypes.

2.3 Sucrose Concentration

Plant cells and tissues in the culture medium are heterotrophic and therefore, are dependent on the external carbon for energy. Sugars have two roles in culture medium, both as carbon source and as osmotic pressure regulator, which are both important for callus induction (Bishnoi *et al*. 2000). The most commonly used sugar in plant tissue culture is sucrose (Reed, 1966).

Sucrose level of 2 % - 5 % was appropriate for rice anther culture (Reinert and Bajaj, 1977). Sucrose concentration above 6 % in the induction medium often increases the proportion of the albino plants (Wang *et al*., 1977). Similarly, Chen (1978) reported that 6 % and 9 % sucrose enhanced both the formation of callus and the organogenesis of early uninucleate microspores, but most of the plantlets cultured in 9 % sucrose were albino.

It is suggested that sucrose can be used for rice plant regeneration because of lower price than maltose and in an amount of 3% in culture medium. Although it is inclined to use sucrose in culture medium, regarding the time interval of callus induction in rice androgenesis which is 3-4 weeks (Yoshida *et al*., 1995) and in this time interval when sucrose is decomposed in culture medium (Last and Brettell, 1990), it is suggested to use more amount of sucrose 60 $g.L^{-1}$ for androgenesis.

2.4 Plant Growth Regulators

Plant growth regulators are important in plant tissue culture since they promote growth, differentiation and organogenesis of plant tissues in cultures. In addition to nutrients, the growth regulators such as auxin, cytokinin, abscisic acid, gibbrellins are mainly added to support growth of tissue. The success of tissue culture can be enhanced by improving the composition of culture medium by manipulating the growth regulators (Mandal and Gupta, 1995).

2.4.1 Auxin

Auxin is one of the most significant regulators of differentiation in most monocot plants (Nishi *et al.,* 1968). The auxin most commonly used in plant tissue culture are 2,4-D (2,4- Dichloro phenoxyacetic acid), NAA (Naphthaleneacetic acid), IAA (Indole 3-acetic acid), IBA (Inole 3-butyric acid). Among the auxin, 2,4-D and NAA are the most widely used growth regulators for induction of callus from rice anthers (Tapia *et al*., 2002). Monirul *et al*., (2004) observed that addition of 1 $mg.L^{-1}$ 2,4-D to callus induction medium improved the callus induction and regeneration potential of the responsive hybrid rice line IR-69690. Skider *et al*., (2006) reported that 2-3 $mg.L^{-1}$ 2,4-D was suitable for callus induction in aromatic rice. Sripichitt and Cheewasestatham (1994) also reported that the callus formation of Khao Dawk Mail rice was optimum (96.3%) when cultured on MS medium supplemented with 2 $mg.L^{-1}$ 2,4-D. N_6 medium supplemented with 2 $mg.L^{-1}$ 2,4-D was found to be suitable for callus induction for Basmati rice cultivars (Naqvi *et al.,* 2005).

Xa and Lang (2011) demonstrated that N_6 medium with 2 $mg.L^{-1}$ 2,4-D and 2 $mg.L^{-1}$ NAA was used as callus induction medium for callus induction in indica x indica crosses hybrid, which gave 5.13-9.27% callus induction and 6.17-14% green plant regeneration when transferred on MS medium with 1 $mg.L^{-1}$ BA and 2$mg.L^{-1}$ Kinetin. Concerning the genotypes and culture medium effects on anther culture response, Mandel and Gupta (1995) expressed that 2,4-D or NAA 2 $mg.L^{-1}$ concentration is effective in callus induction and calli which were induced in culture medium containing NAA had more green plant regeneration frequency than calli which were induced in culture medium containing 2,4-D.

MS medium supplemented with 0.5-2 $mg.L^{-1}$ 2,4-D was suitable for callus formation in embryos of Australian rice varieties (Azria and Bhalla, 2000). IAA and NAA may induce direct androgenesis while 2,4-D promotes rapid cell proliferation

and formation of nonembryogenic callus (Ball *et al*., 1993). Moreover, 2,4-D results in high callus induction and the 2,4-D induced calli produce higher green plant than the NAA induced calli. Moreover, 2,4-D inhibits the organogenesis of calli and NAA promotes the formation of roots and sometimes completes plants (Martin *et al.,* 1982). In tissue culture, auxin is usually used to stimulate callus production and cell growth, to initiate shoots and rooting, to induce somatic embryogenesis, to stimulate growth from shoot apices and shoot stem culture.

2.4.2 Ratio of auxin and cytokinin

The relative concentrations of the growth factors namely auxin and cytokinin are crucial for the morphogenesis of culture systems. When the ratio of auxins to cytokinins is high, embryogenesis, callus initiation and root initiation occur. On the other hand, for axillary and shoot proliferation, the ratio of auxins to cytokinins is low. For all practical purposes, it is considered that the formation and maintenance of callus culture requires both auxin and cytokinin, while auxin is needed for root culture and cytokinin for shoot culture. The actual concentrations of the growth regulators in culture media are variable depending on explants type and the plant species.

The combinations of hormone type and concentration of hormones can greatly affect the development of microspores and impact the morphogenetic proceed leading to the production of the plants (Trejo-Tapia *et al*., 2002). Ball *et al*., (1993) stated that the type and concentration of growth regulators can be the deciding factor for pollen embryogenesis. Both auxin and cytokinin are crucial constituents in rice anther culture medium, control the differentiation and dedifferentiation processes in *in vitro* culture. 2,4-D as only hormone for in vitro development of rice microspores and hence it was included in the culture medium either singly or in combination with cytokinin with other auxins (Iyer and Raina, 1972).

Among the synthetic auxins sources 2,4-D and NAA were commonly used for callus induction from rice anthers and auxins are the most essential growth regulators required for induction of callus from anthers of cereals (Zhu *et al*., 1998). Chen *et al*., (1991) reported that callus forming ability from anthers of rice was high in medium supplemented with 2,4-D, but the regeneration ability from these calli was low as compared to calli formed on medium supplemented with NAA.

MS medium with 2 $mg.L^{-1}$BAP, 1 $mg.L^{-1}$ kinetin and 1 $mg.L^{-1}$ NAA gave the maximum green plant regeneration (8.95%) in Boro hybrid rice (Sen *et al*., 2011). In

addition, Shahnewaz *et al*., (2003) also observed that MS medium supplement with 1 $mg.L^{-1}$ Kinetin, 1 $mg.L^{-1}$ NAA and 1 $mg.L^{-1}$ BAP gave the green plant regeneration in haploid rice plant.

The best shoot responses on MS medium supplemented with 1 $mg.L^{-1}$ NAA and 1 $mg.L^{-1}$ BA (Marasi *et al*., 1996). Pandey *et al*., (1994) found that 2 and 3 $mg.L^{-1}$ IAA and Kinetin produced the most shoots in rice anther culture. Recent studies observed that the highest green shoot regeneration from callus of anther was from N_6 medium supplemented with 1 $mg.L^{-1}$ NAA and 2 $mg. L^{-1}$BAP. The anther derived callus were cultured on MS supplemented with 0.5 $mg.L^{-1}$ NAA, 0.25 $mg.L^{-1}$ IAA, 1 $mg.L^{-1}$ Kinetin and 0.5 $mg.L^{-1}$ BAP for plant regeneration in indica rice genotypes (Li *et al.,* 2011).

Many reports have been described on the successful regeneration of anther derived callus from medium supplemented with 2,4-D. Auxin in high concentrations will prevent green plant regeneration (Mandal and Gupta, 1995). Raina and Zapata (1997) reported that 2,4-D has proven to be a potent auxins for callus induction from cultured anthers, but the regeneration ability of callus induced under high 2,4-D levels is poor, especially for indica rice, in comparison to callus induced on medium with lower 2,4-D levels. Sah (2008) reported that higher rate of albino plant production might be attributed to higher rates of 2,4-D in the media.

2.5 Factors Affecting on Success of Anther Culture

2.5.1 Genotype

The selection of plant material for an anther culture technique is important. In particular, genotype plays an important role in determining the success or failure of the *in vitro* androgenesis. Genotype is one of the deciding factors for anther response. Anther culture of rice is influenced by the genotypes of the explants (Li, 1991) and general trend has been reported as follows: japonica x waxy > japonica x japonica > japonica > indica x japonica > indica x indica.

Most of the *in vitro* morphogenesis responses are genotype dependent (Bhojwani and Razdan, 1996). In general, indica cultivars of rice exhibit poorer androgenic response than the japonica cultivars (Hu, 1985 and Raina, 1997). Merit *et al*., (1987) demonstrated that anther culture response varied from 41% for japonica cultivar to 0 % for indica cultivar.

Many researcher had observed that the variability in anther culture performance among parents and their hybrids plant. Thuan *et al*., (2001) observed that anthers forming callus was the highest in Khao Hom Suphanburi x DS15 cross. The callus formation revealed that anthers from OM5992 x OM4900 cross produced the highest number of calli (9.27%) and the lowest number of calli inducted OM3536 x OM4900 (5.13%) by Xa *et al*., (2011). There are strong genotypic effects on callus induction and green plant regeneration rates varied with genotypes. Gioi (2004) found that anther culture of F_1 plants from crosses between IR64 and new plant types cultivars reached the highest green plant regeneration 5.72%.

Anther of F_1 hybrid is an excellent material to produce haploids in crop improvement of breeding program. Previous studies have showed that the anther ability of F_1 hybrids and F_2 plants were better than parents. Androgenesis in indica x Basmati rice hybrids, high plant regeneration frequencies were observed from microspore derived calli of some of F_1 hybrids and F_2 plants as compared to their actual parents (Bishnoi *et al*., 2000).

Genotype affected callus induction, green plant regeneration and culture efficiency. For its effective utilization in breeding program, the haploid production technique should allow genotype-independent production of large numbers of haploids. Moreover, Chen *et al*., (1991) also reported that frequency of anthers producing callus, capacity of callus to differentiate plants and chromosome number of regenerated plants are related to the genotype of the plant providing the anthers.

2.5.2 Physiological condition of donor plants

The physiological state of donor plant is affected by several factors on the androgenic response of microspores. In most species, the best response usually comes from the anthers obtained from the first flush of flowers produced by a plant.

The growth conditions of the donor plants have significant effect on the yield of androgenic pollen in rice. The plants of IR43 that reached the panicle emergence stage under long days (>12 h), high solar radiation (>18Mj m-2) and sunshine (>8 h) and day/night temperature (34ºC/24ºC) showed highest anther culture response. They also observed that the plants grown in the field were significantly superior than those grown in the glasshouse or in pots near the field. Superiority of field grown plants over glasshouse-grown plants has also been reported for other cereals, including maize (Petolino and Thompson, 1987) and wheat (Lu and Lai, 1991).

Various environmental factors exposed to the donor plants may also effect haploid plant production. These factors have been found to influence the number of plants produced from anther culture. Alternations in the physiology of the donor plant by other treatments such as additional salts (tobacco microspore) or 2-chloroethyl phosponic acid for 48 h at 10ºC (rice inflorescence) have been shown to affect androgenesis (Heberle and Reinert, 1979 and Wang *et al*., 1973).

2.5.3 Duration of pre-treatment on explant

Application of stresses on explant, such as temperature treatment, osmotic shock and sugar starvation during the developmental period of pollen grains is known to be essential for the induction of androgenesis in several plants, including cereals (Bhojwani and Razdan, 1996). Low temperature shock has been reported to enhance the androgenic response in several species including rice (Ogawa *et al*., 1995, Gueye and Nidr, 2010 and Sen *et al*., 2011). However, the type, duration and the time of application of these pre-treatments may vary with the species or even variety (Datta, 2001).

The most widely used pre-treatment for androgenesis is the low temperature shock. In rice androgenesis, panicles were given a cold pre-treatment but the temperature and duration varied. Ogawa *et al*., (1995) observed that 28 days of pre-treatment at 10^{o}C was optimum for indica. Gupta and Borthakur (1987) selected pre-treatment at 10^{o}C for 11days for anther culture response of the indica cultivar.

Although the frequency of callusing after cold-treatment (25 days) was fairly high, most of the plants regenerated from the calli after long cold pre-treatment were albinos. Pande (1999) observed that cold pre-treatment 10^{o}C for 10 days was most suitable for anther cultures of the indica rice genotype, IR43 and pre-treatments longer than 11 days resulted in albino production. Reddy *et al*., (1985) reported that a brief (10 min) exposure to high temperature (35^{o}C) before cold-treatment was better for pollen callusing but it adversely affected green plant production.

2.5.4 Stage of microspore development

The most critical factor affecting haploid production from anther culture is the stage of microspore development. In general, optimum response was obtained in tobacco from anther cultured during uninucleate to early binucleate microspore (Reed, 1966). Niizeki and Oono (1971) first reported in rice that a specific developing stage or the stage if uninucleate pollens was effective in culture. Chen (1977) also found

that callus initiation occurred with highest frequency in anthers containing mid-uninucleate microspores. The callus derived from various stages of microspore development differed in the potential to differentiate into plants.

2.5.5 Composition of culture medium

Culture media plays an important role in success of anther culture in *in vitro*. Many culture media were widely used for plant tissue culture. Among them, N_6 medium has been most widely used for rice anther culture (Raina, 1997). Reddy *et al.,* (1985) studied that 8 indica cultivars found He_2 medium to be better than N_6 medium. SK_1 medium was half as effective as N_6 medium so far as the frequency of pollen callusing is concerned but the calli formed on this medium produced twice as many green plants as those on N_6 medium.In addition, Herath *et al.,* (2007) demonstrated that the highest callus induction of F_1 hybrids was observed in N_6 medium. N_6 medium used mainly for japonica rice and for indica rice is not suitable (Gosal *et al*., 1997). Callus formation medium had also a role in plant regeneration, each callus culture medium will have the best reaction with a specific regeneration medium.

2.5.6 Culture condition

Temperature is one of the important factors of androgenesis that influence the induction of pollen and callus development. Anther culture is usually incubated at 25 ±1ºC. Dunwell (1983) reported that there are several explanations on the success of androgenesis related to temperature. In rice anther culture, high temperature may disrupt the normal development of somatic anther tissue, synchronize the microspore population, and increase the total number of spores at the stage of cell cycles that is susceptible to induction. Additionally, the high temperature may result in increased growth rate of haploid embryos when compared to nonhaploids in rice (Jain *et al*., 1996). Some species respond best when exposed to alternating light and dark periods.

CHAPTER III
MATERIALS AND METHODS

3.1 Experimental Site and Duration

The experiment was carried out at Field and Plant Tissue Culture Laboratory, Department of Horticulture and Agricultural Biotechnology, Yezin Agricultural University, Yezin, Nay Pyi Taw, from January 2015 to October 2016. Hybridization program was done from April to November 2015. The anther culture of parents and their F_1hybrids was done from March to June 2016.

3.2 Materials

Five indica rice genotypes were selected as parental lines for hybridization based on callusing ability of anther culture, which were provided by Seed Bank, Department of Agricultural Research (DAR), Yezin, Nay Pyi Taw. Five selected indica rice genotypes and their F_1 progenies were used for anther culture.

No.	Genotypes
1	Shwe Man -1
2	Sin Thwe Latt
3	Sin Akari-3
4	Thee Htat Yin
5	Yar-8

3.3 Methods

3.3.1 Hybridization

Crossing among the selected rice genotypes was conducted during March to May 2015. Five F_1 hybrids were produced. F_1 hybrid seeds were harvested in 30 days after pollination and were stored in glassine bags before germination.

No.	Crosses
1	Shwe Man-1 x Thee Htat Yin
2	Thee Htat Yin x Sin Thwe Latt
3	Sin Thwe Latt x Sin Akari -3
4	Yar 8 x Thee Htat Yin
5	Sin Thwe Latt x Yar-8

3.3.2 Planting Seeds

For parental lines, about 150 seeds of each parental genotype were germinated in trays. The twenty days old seedlings were transferred to field. For F_1 hybrids, seeds of the each cross were treated in benomyl fungicide solution for 6 hours and then transferred wash with water and soak until the seeds are germinated. F_1 hybrid seedlings were transferred into plastic pots. After 2 weeks, these plants were transferred to the field. They were provided with a regular supply of water and recommended cultural practices under intensive care and management.

3.3.3 Steps for rice anther culture

(a) Panicles collection

Panicles were collected from the healthy plants of the parents and F_1 hybrids during 8:00-10:00 a.m when the distance between flag leaf and penultimate leaf was 5-12 cm depending on genotypes. Leaf blades of the collected panicles were removed from the stem with scissors.

(b) Panicles sterilization

After collecting the panicles, they were sterilized with standard sterilization protocol for rice panicles. Firstly, the selected panicles were washed thoroughly with sterilized water. Secondly, they were sterilized with 95% ethanol for 30 seconds. And then, these panicles were wrapped in aluminium foil and covered with black plastic sheet. Finally, they were kept in low temperature incubator at 10ºC for 8-10 days for cold treatment.

(c) Cytological examination

The anthers were squeezed out in petridish with the help of the forceps and the stage of pollen grains was examined using microscope. The extracted anther were mixed in Acetic acid: Chloroform: Ethanol (1:6:1) solution for 24 hours. The early uninucleate microspore is lightly staining with a centrally located nucleus .As the nucleate microscope develops, a large central vacuole was formed .As the binucleate microscope stages, the intensity of the staining increases and starch granules begin to accumulate.

(d) Anther inoculation

Before inoculating the anthers, the cold treated panicles were sterilized in Clorox (20%) for 25 minutes, followed by rinsing three times thoroughly with double distilled water and then decant the water. The spikelets in which anthers developed at early uninucleate to early binucleate stage were aseptically excised in petridish containing sterilized water to prevent desiccation. The anthers were squeezed out in test tube using forceps. About 120 anthers were cultured in each test tube containing about 10 ml culture media. The cultured test tubes were covered with aluminum foil and labeled.

3.3.4 Culture media

(a) Callus induction medium

Five selected indica rice genotypes were tested for callus induction medium to know their ability to produce callus by using N_6 (Nitchs') medium (Chu, 1975) with 2 $mg.L^{-1}$ 2,4-D and 0.5 $mg.L^{-1}$ Kinetin. The medium was adjusted to pH 5.8. According to the preliminary experiment, N6 medium was used for the respective F_1 hybrids.

(b) Plant regeneration medium

Calli (1-3 mm) of each genotype were precultured on 'M' shaped- filter paper bridges with MS (Murashige and Skoog, 1962) liquid medium with 2 $mg.L^{-1}$ 2,4-D and 0.5 $mg.L^{-1}$ Kinetin. After 2 weeks, the calli from the liquid medium were transferred onto the MS medium with supplemented 1 $mg.L^{-1}$ NAA, 1 $mg.L^{-1}$ IAA, 1 $mg.L^{-1}$ BAP and 2 $mg.L^{-1}$ kinetin. The pH of medium was adjusted to 5.7.

3.3.5 Culture condition

Cultured anthers were incubated in dark condition at 25 ± 2ºC till callus induction. Anther culture response was observed for callus formation for 3 months. The regenerated calli were observed till green plant formation under 16/8 light/ dark hours at 25 ± 2ºC.

3.3.6 Acclimatization of anther-derived rice plants

The completely regenerated plants were transferred in Yoshida solution for stronger root formation for 2 weeks. After 2 weeks, well rooted plants were acclimatized to sterile paddy soil in plastic cup for 2 weeks before growing in field condition. The anther-derived rice plants were grown individually in each plastic pot under open condition.

3.4 Data Collection

The following data were collected during the development of callus induction and plant regeneration.

(1) Number of days to induce callus (day)

Day to induce callus formation was counted first day to form callus from anther of each genotype after inoculation.

(2) Number of callus

The number of callus was recorded by counting the callus producing from each genotype.

$$\textbf{(3) Callus induction (\%)} = \frac{\text{Total number of calli produced}}{\text{Total number of anther plated}} \times 100$$

(4) Callus type

Calli which have compact and friable type were recorded before transfer to regeneration medium.

(5) Callus color

Calli which have white, brown and light yellow color were recorded before transfer to regeneration medium.

(6) Days to form green spot

The days to form green spot from regenerated callus were counted.

(7) Green plant formation (%)

$$\text{Green plant formation \%} = \frac{\text{Total number of calli production green calli}}{\text{Total number of calli plated}} 100$$

(8) Albino plant formation (%)

$$\text{Albino plant formation \%} = \frac{\text{Total number of calli production albino calli}}{} 100$$

(9) Number of green plant

The number of green plant was counted the green plants inducing of green points from callus of each genotype.

(10) Survival (%)

Survival rate of anther-derived plants was recorded during adaptation period.

(11) Panicle status

The panicle of the anther-derived plants which have sterility or fertility status was recorded.

3.5 Data Analysis

The data for callus induction and green plant regeneration were collected and computed to find out callus induction and green plant regeneration percentage. The Completely Randomized Design (CRD) was used for statistical analysis of both variations. Mean value of callus induction and green plant regeneration ware calculated using Microsoft Excel (2010).

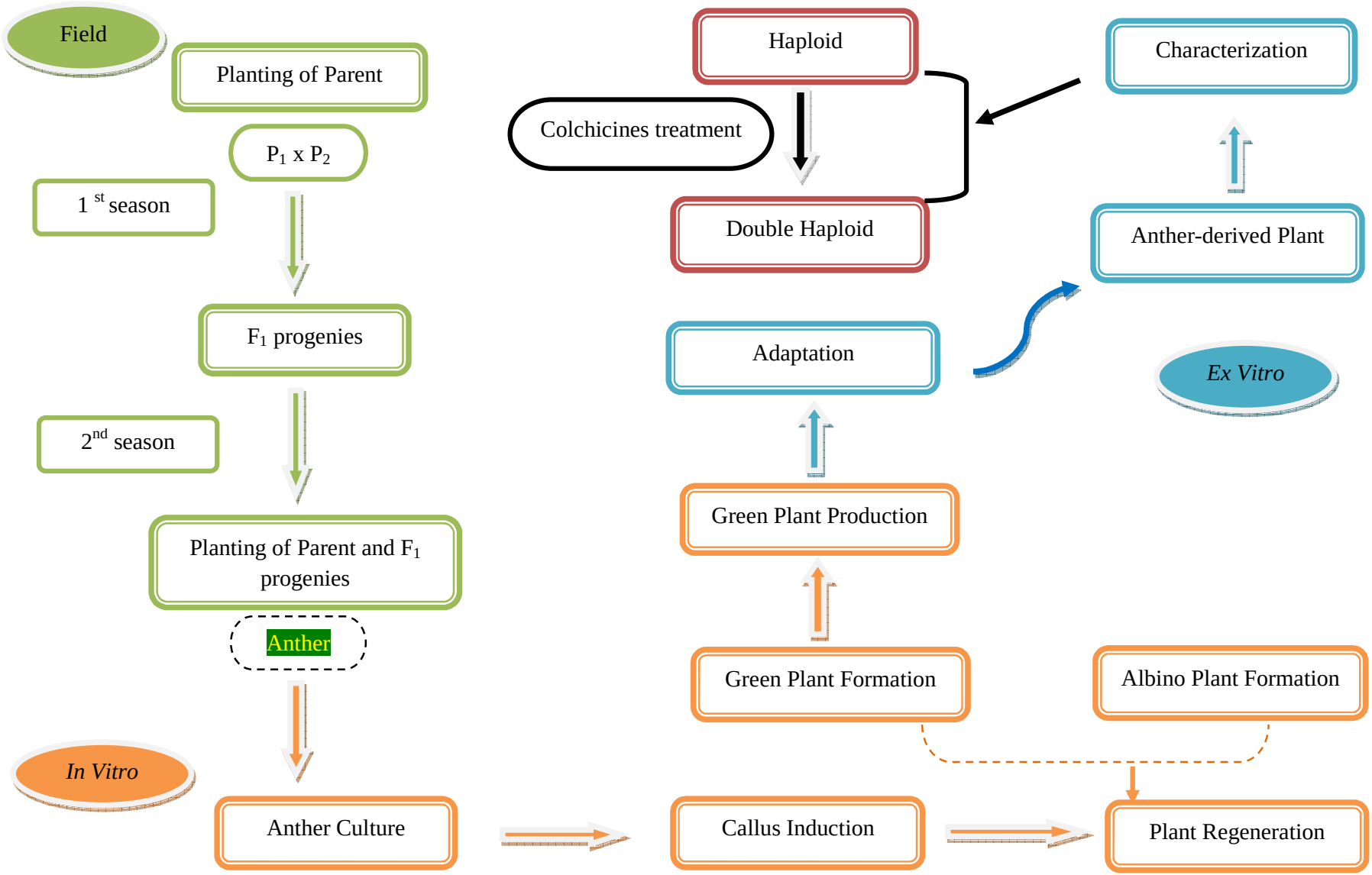

Figure 3.1 Flow Schematic of Rice Anther Culture

CHAPTER IV
RESULTS AND DISCUSSION

4.1 General Description of Callus Induction and Plant Regeneration

Anthers of all genotypes changed color from yellow to brown and then into dark brown within 1-4 weeks after inoculation (Figure 4.1.a). The change into dark color may be due to the accumulation of toxic products resulting from phenolic oxidation. Blackening occurred through the action of copper-containing oxidase enzymes such as polyphenoloxidases and tyrasinose which oxidized phenols to quinonse (Manaco *et al.*, 1979). Therefore, the dark brown color formation in callus may be due to these quinones. The responsive anthers showed slight swelling around it and subsequently induced callus (Figure 4.1.b). The anthers of the parental indica rice and F_1 hybrids genotypes produced callus within two months after inoculation (Figure 4.1.c). Calli derived from all genotypes were transferred to regeneration medium for green plant regeneration. Green spots appeared from the regenerated calli within 4-10 days (Figure 4.1.d). Although some genotypes started shoot development earlier than root development, some produced only roots. Protocol for androgenesis of rice was shown in Figure 4.7.

4.2 Callus Induction

4.2.1 Number of days to induce callus

Most anthers of selected indica rice and F_1 hybrids genotypes formed callus induction within 4-8 weeks in Table 4.1. The anthers of five selected indica rice and five F_1 hybrid combinations were inoculated on N_6 medium for callus induction. Anthers of all parental indica rice genotypes exhibited callus within 5 weeks after inoculation. The earliest day (28 days) to induce callus was found in Sin Akari-3 among parents. Shwe Man-1, Sin Thwe Latt and Yar-8 genotypes produced callus within 5 weeks and Thee Htat Yin gave the callus induction approximately 6 weeks.

Anthers of four F_1 hybrid crosses produced calli within 5-8 weeks after inoculation, the remaining one showed no response on callus induction. Among crosses, Shwe Man-1 x Thee Htat Yin, Yar-8 x Thee Htat Yin and Sin Thwe Latt x Yar-8 produced callus within 4 weeks. The days to induce callus of these three crosses were nearly the same. The longest days to induce callus was found in Thee Htat Yin x Sin Thwe Latt. However, Sin Thwe Latt x Sin Akari-3 did not produced callus after 3 months culture.

It was observed that the days to induce callus was varied with the genotypes. These results contradict the report of Wang *et al*., (2011) who reported that the calli of indica x indica hybrids were formed within 6 weeks after incubation. Therefore, this variation may be due to the genotypes used. Reddy *et al*., (1985) and Abe (1992) suggested that the callus forming abilities from rice anther culture and time required for callus induction depend on genotypes. Moreover, Herath *et al*., (2007) documented that the time requirement for callus initiation was genotype dependent.

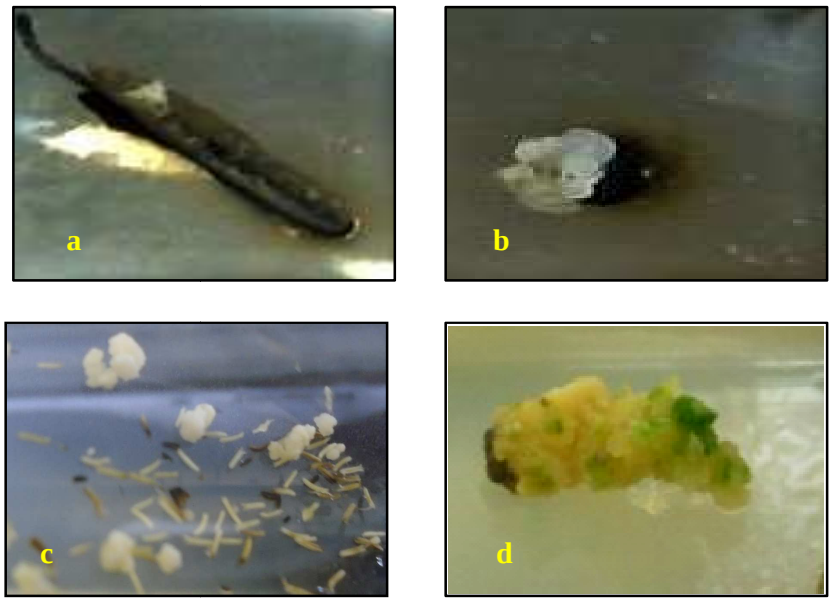

Figure 4.1 Callus induction and plant regeneration (a) anther browning, (b) callus initiation from responsive anther (c) callus formation, and (d) green spot formation from regenerated calli

Table 4.1 Days to induce callus for five selected indica and F_1 hybrids rice genotypes after 3 months inoculation

No.	Genotypes	Days to induce callus
1	Shwe Man-1	34
2	Sin Akari-3	28
3	Sin Thwe Latt	39
4	Thee Htat Yin	43
5	Yar-8	36
6	Shwe Man-1× Thee Htat Yin	32
7	Thee Htat Yin × Sin Thwe Latt	55
8	Sin Thwe Latt × Sin Akari-3	No response
9	Yar-8 × Thee Htat Yin	30
10	Sin Thwe Latt × Yar-8	34

4.2.2 Callus type

Anther-derived calli of all genotypes were compact or friable (Table 4.2). The development of callus was shown in Figure 4.2. The texture of anther-derived calli of Shwe Man-1 and Yar-8 genotypes were friable callus type among parents. The calli texture of Sin Akari-3, Sin Thwe Latt and Thee Htat Yin genotypes were compact. The calli of three crosses; Shwe Man-1 x Thee Htat Yin, Yar-8 x Thee Htat Yin and Sin Thwe Latt x Yar-8 were friable type, the calli of Thee Htat Yin x Sin Thwe Latt were compact. In this observation, it was noticed that some genotypes with friable calli could produce green plant regeneration.

This finding was the same with the finding of Thanh *et al*., (2011) who observed that the callus with friable and light yellow color gave the green plant regeneration. There were two types of calli to regenerate green plant depend on light condition in rice anther culture. Calli formed under light condition were mostly embryogenic which have creamy, dry and compact appearance, while nonembryogenic calli with white, wet and friable characters were found predominantly under dark condition. Therefore, the calli from the anther culture maintained under dark condition were nonembryogenic. However, Siriwardana and Nabors (1983) documented that embryogenic callus displayed higher frequency of plant regeneration than the non-embryogenic. The callus cultured under light condition showed higher proliferation and plant regeneration because light induces morphogenesis process and green spot formation of callus (Janet and Seabrook, 1980).

4.2.3 Callus color

Calli which have 1-3 mm size were transferred to Liquid MS medium. After 2 weeks, all calli were transferred onto solid MS medium. During this period, some calli responded to brown, white and light yellow color (Figure 4.3). The response calli with light yellow or white color turned to green color which can produce green shoots or albino shoots at 3-4 weeks after transferring onto semisolid MS medium. It was noted that all green calli could not produce green shoots. Premvaranon *et al., (*2011) reported that only light green color gave the green plant regeneration in anther culture of indica hybrids rice genotypes. However, Skider *et al*., (2006) observed that the color of call us was whitish which can produce green plant in japonica type, aromatic rice.

Table 4.2 Callus type and callus color of callusing responsive genotypes

No.	Genotypes	Callus type	Callus color
1	Shwe Man-1	Friable	White
2	Sin Akari-3	Compact	White
3	Sin Thwe Latt	Compact	Brown
4	Thee Htat Yin	Compact	White
5	Yar-8	Friable	Light yellow
6	Shwe Man-1× Thee Htat Yin	Friable	White
7	Thee Htat Yin × Sin Thwe Latt	Compact	Brown
8	Yar-8 × Thee Htat Yin	Friable	White
9	Sin Thwe Latt × Yar-8	Friable	White

Figure 4.2 Callus type (a) compact and (b) friable

Figure 4.3 Callus color (a) brown, (b) white, and (c) light yellow

4.2.4 Callus induction percentage

Callus induction started within 2 months of culture and was observed in both selected indica rice genotypes and F_1 hybrids. The callus induction percent of selected indica rice and F_1 hybrids are showed in Table 4.3. The percentage of callus induction varied from 0.1-2.6% among genotypes. Among parent, Yar-8 genotype produced the maximum callus induction (2.6%), followed by Shwe Man-1 (1.5%), Sin Akari-3 (0.8%), Sin Thwe Latt and Thee Htat Yin (0.5%) on the same medium. It was found that callus induction of anthers varied with the different genotypes. Therefore, this finding agrees with those of Medhabati (2014) who observed that the callus induction varied with the different genotypes on the same medium.

The callus induction of F_1 hybrids was 0-1.8%. Yar-8 x Thee Htat Yin gave the highest callus induction (1.8%), followed by Shwe Man-1 x Thee Htat Yin (0.9%), Sin Thwe Latt x Yar-8 (0.7%) and Thee Htat Yin x Sin Thwe Latt (0.1%). However, Sin Thwe Latt x Sin Akari-3 showed no callus induction. Cross of Yar-8 x Thee Htat Yin showed the best callus induction among crosses. In this case, the genotypes as female parents (Yar-8 and Shwe Man-1) showed the best response on callus induction, but Thee Htat Yin showed the lowest callus induction among parents.

In this experiment, it was observed among indica rice genotypes that the less response genotypes can increase callusing ability of anther culture by crossing with the high response genotypes. The selection of genotypes for callus induction in rice anther culture is very important. Narasimman and Rangasamy (1993) stated that both callus induction and green plant regeneration have varied depending on the specific genotypes used to construct the hybrids. In addition, Imuta *et al.,* (1991) reported that callus induction ability of anther culture varied with different varieties. In the present study, similar results were observed that rice genotypes showed differently to produce calli on the same callus induction medium.

Table 4.3 Callus induction of selected indica and F_1 hybrids rice genotypes

No.	Genotypes	No. of callus	callus induction %
1	Shwe Man-1	26	1.5
2	Sin Akari-3	9	0.8
3	Sin Thwe Latt	14	0.5
4	Thee Htat Yin	8	0.5
5	Yar-8	46	2.6
6	Shwe Man-1× Thee Htat Yin	16	0.9
7	Thee Htat Yin × Sin Thwe Latt	2	0.1
8	Sin Thwe Latt × Sin Akari-3	0	0.0
9	Yar-8 × Thee Htat Yin	32	1.8
10	Sin Thwe Latt × Yar-8	13	0.7

4.3 Plant Formation

4.3.1 Number of days to form green spot

Calli with friable and compact type were regenerated in MS medium for plant formation under light condition. Some calli differentiated only into green plant formation or albino plant formation. Table 4.4 shows that the days to form green spot from regenerated calli of callusing response genotypes. It can see clearly that only four genotypes of all genotypes induced green region (green spot) within 2-4 weeks after transferring of callus. The earliest day to form green spot was observed in Yar-8, followed by Shwe Man-1 genotype. The calli of F_1 hybrids (Shwe Man-1 x Thee Htat Yin and Yar-8 x Thee Htat Yin) exhibited green spot from the regenerated calli within 2 weeks. Other genotypes were cultured on regeneration medium for inducing green spot untill 4 weeks after transferring of callus. However, it was observed no response on green spot formation. Some researchers found that the green spot formation from regenerated calli of indica rice genotypes occurred within 1-3 weeks (Thanh *et al.*, 2006 and Medhabati *et al.*, 2014).Therefore, the formation of green spot from regenerated calli was mainly depend on age of callus and fresh of callus. Wang *et al.*, (1977) and Chen *et al.*, (1986) also reported that the green plant regeneration is greatly influenced by age and size of callus.

4.3.2 Green plant formation

Green plant formation from regenerated calli of callusing response rice genotypes was shown in Table 4.5. Out of five parents, only two genotypes gave the green plants. Yar-8 genotype gave the maximum green plant formation (37%). The remaining three genotypes did not produce green plants. Among crosses, only two crosses, Yar-8 x Thee Htat Yin and Shwe Man-1 x Thee Htat Yin, exhibited 9.4 % and 6.3% green plant formation. Although callus induction was found in almost tested genotypes, green plant formation was found in only four genotypes. It was found that there were strong genotypic effects in green plant formation. Although Thee Htat Yin did not induce green plants, crosses of Shwe Man-1 x Thee Htat Yin and Yar-8 x Thee Htat Yin can produce green plants. It means that the green plant formation ability was dependent on their corresponding parental genotypes. Moreover, Sree *et al.*, (1992) also reported that green plant formation of anther culture varied greatly with genotypes.

Table 4.4 Days to form green spot from regenerated calli of responsive genotypes

No.	Genotypes	Days to induce green spot
1	Shwe Man-1	11
2	Sin Akari-3	No response
3	Sin Thwe Latt	No response
4	Thee Htat Yin	No response
5	Yar-8	8
6	Shwe Man-1× Thee Htat Yin	15
7	Thee Htat Yin × Sin Thwe Latt	No response
8	Yar-8 × Thee Htat Yin	14
9	Sin Thwe Latt × Yar-8	No response

Table 4.5 Green plant formation from regenerated calli of responsive genotypes

No.	Genotypes	No. of green calli	Green plant formation (%)
1	Shwe Man-1	5	19.2
2	Sin Akari-3	0	0.0
3	Sin Thwe Latt	0	0.0
4	Thee Htat Yin	0	0.0
5	Yar-8	17	37
6	Shwe Man-1× Thee Htat Yin	1	6.3
7	Thee Htat Yin × Sin Thwe Latt	0	0.0
8	Yar-8 × Thee Htat Yin	3	9.4
9	Sin Thwe Latt × Yar-8	0	0.0

4.3.3 Albino plant formation

Table 4.6 shows that albino plant formation from regenerated calli of callusing response genotypes. Six genotypes produced the albino calli and albino plants among the tested genotypes. In all genotypes, Thee Htat Yin x Sin Thwe Latt (50%) produced the largest amount of albino plant formation. The albino plant formation was found in Shwe Man-1 (15.4%), Sin Akari-3 (14.3%) and Yar-8 (10.9%) respectively in parents. However, calli of Sin Thwe Latt and Thee Htat Yin could not induce albino plant. Sin Thwe Latt x Yar-8 and Yar-8 x Thee Htat Yin gave 7.7% and 3.1% albino plants formation. Calli of Shwe Man-1 x Thee Htat Yin did not produce albino plant regeneration. In this result, it was noted that Yar-8 and cross of Yar-8 x Thee Htat Yin genotypes produced low amount of albino plant formation. Formation of albino plants is a major problem in rice anther culture especially in indica rice. According to Roy and Mandal (2005), green plants formation from androgenic calli is very low and high percent of albino plant formation are the principle constraints in successful anther culture in rice. Moreover, the albino formation depended on the varieties used to construct hybrids and anther pretreatment temperature.

Table 4.6 Albino plant formation from regenerated calli of responsive rice genotypes

No.	Genotypes	No. of albino calli	Albino plant formation (%)
1	Shwe Man-1	4	15.4
2	Sin Akari-3	2	14.3
3	Sin Thwe Latt	0	0.0
4	Thee Htat Yin	0	0.0
5	Yar-8	5	10.9
6	Shwe Man-1× Thee Htat Yin	0	0.0
7	Thee Htat Yin × Sin Thwe Latt	1	50.0
8	Yar-8 × Thee Htat Yin	1	3.1
9	Sin Thwe Latt × Yar-8	1	7.7

4.3.4 Green plant production

Only three genotypes produced green plant among the tested genotypes. Most of the green regions developed multiple green shoots as shown in Figure 4.4. Therefore, shoot clusters were subcultured for two times. In this observation, most of genotypes produced shoot formation earlier than root formation. Some genotypes produced only root formation. This observation may be due to hormone application for regeneration medium and delay transferring of callus from callus induction medium to green plant regeneration medium.

Table 4.7 shows the green plant production of response genotypes. Yar-8 and Shwe Man-1 genotypes can produce green plant among parents. The highest green plant production was observed in Yar-8 genotype. Among crosses, only the cross of Yar-8 x Thee Htat Yin genotype showed green plant production. This result pointed out that different genotypes respond differentially to green plant production.

4.3.5 Survival rate of transplanted anther-derived plants

Anther-derived plantlets were individually transferred into the plastic pots. It was shown in Table 4.8 that all transplanted anther-derived plants had 100 % survival rate. This finding was a little variation with other research findings. A survival rate of anther derived plant was reported 50-75 % and 87 % (Herath *et al.*, 2007 and Wang *et al.*, 2011). In plant tissue culture, adaptation processes is very important for *in vitro*-derived plants to survive before transfer to natural environment. Therefore, it can be assumed that this variation may be due to adaptation process. In this study, the adaptation process was done well (Figure 4.5).

Table 4.7 Green plant production of response rice genotypes after 2 times subculture

Genotypes	No. of green plant from regenerated calli	No. of green plant	
		Subculture 1	Subculture 2
Shwe Man -1	3	5	6
Yar-8	15	35	40
Yar-8 × Thee Htat Yin	8	13	14

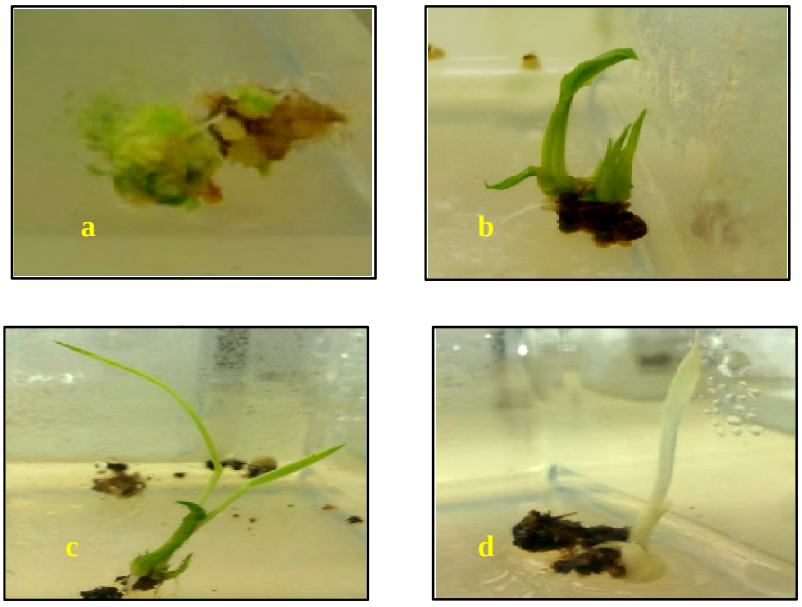

Figure 4.4 Green plant production (a) green region of regenerated callus, (b) green shoot formation, (c) green plant, and (d) albino plant

Table 4.8 Survival rate of transplanted anther-derived plants

Genotypes	Transplanting survival		
	Plant transferred	Plant survived	survival %
Shwe Man -1	6	6	100
Yar-8	40	40	100
Yar-8 × Thee Htat Yin	14	14	100

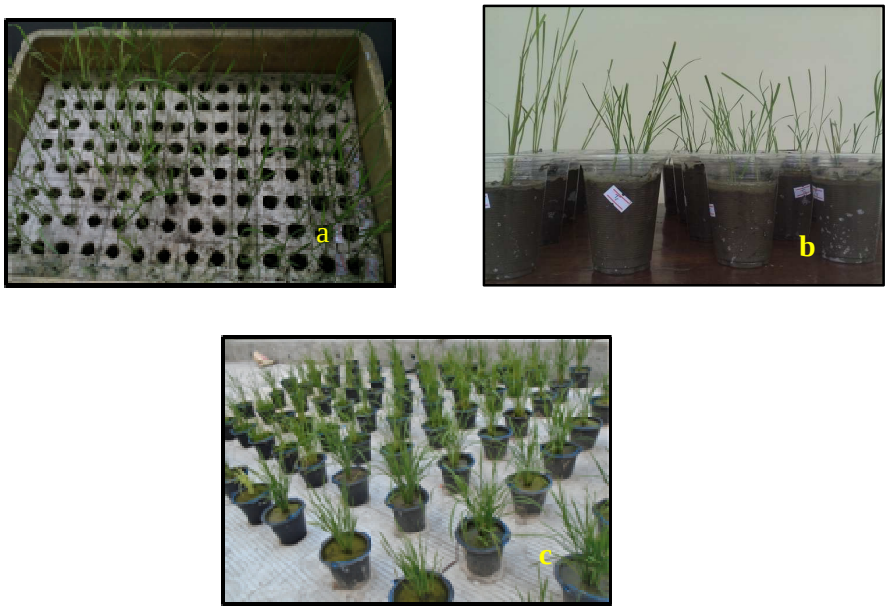

Figure 4.5 Adaptation process anther-derived plants (a) Yoshida solution for 2 weeks, (b) well rooted plants in paddy soil, and (c) plants under natural environment

4.3.6 Characterization of anther-derived plants by panicle status

Several methods are available for determining the ploidy level of regenerated anther-derived plants. According to (Mishra *et al.,* 2015), ploidy levels based on morphological characteristics of the anther-derived plants, revealed that the haploid plants were fully sterile and double haploid plants were fully fertile. In this observation, the plants derived from anther may be haploid or double haploid plants. All panicles of Yar-8 anther-derived plants were sterile (Figure 4.6. c). Although some panicles of Shwe Man-1 and cross of Yar-8 x Thee Htat Yin anther-derived plants were fertile (Figure 4.6. a and b), some panicles were sterile.

It can be clearly seen in Table 4.9 that Yar-8 plants may be assumed as haploid plants due to fully sterile panicles and some of Shwe Man-1 and Yar-8 x Thee Htat Yin genotypes plant were probably double haploid plants. Hence, identification of haploid plants is needed for double haploid production. Chromosome complement is therefore necessary. The most frequently used application is treating with colchicines (Jake *et al*., 2003). The haploid plants can be treated with colchicines solution by doubling chromosome to get double haploids.

The finding of this experiment is in the aggrement of Mishra *et al.,* (2013) who reported that rice is a unique material in which around 30-40 % of the anther-derived plants are double haploids due to the spontaneous doubling of the haploids. Therefore, further experiment is needed to address the confirmation of double haploid lines of the anther-derived plants with fertile panicle by cytological examination and molecular marker technology. Haploid plants of Yar-8 genotype have the potential to produce double haploid lines by treating with colchicines solution.

Table 4.9 Characterization of ploidy levels for anther-derived plants by panicle status

Genotypes	No. of anther-derived plants	Ploidy level of anther-derived plants	
		Haploid	Double haploid
Shwe Man-1	6	2	4
Yar-8	40	40	0
Yar-8 × Thee Htat Yin	14	4	10

Figure 4.6 Panicle status of anther-derived plants (a) Fertile panicle of Shwe Man-1, (b) Fertile panicle of Yar-8 x Thee Htat Yin, and (c) Sterile panicle of Yar-8

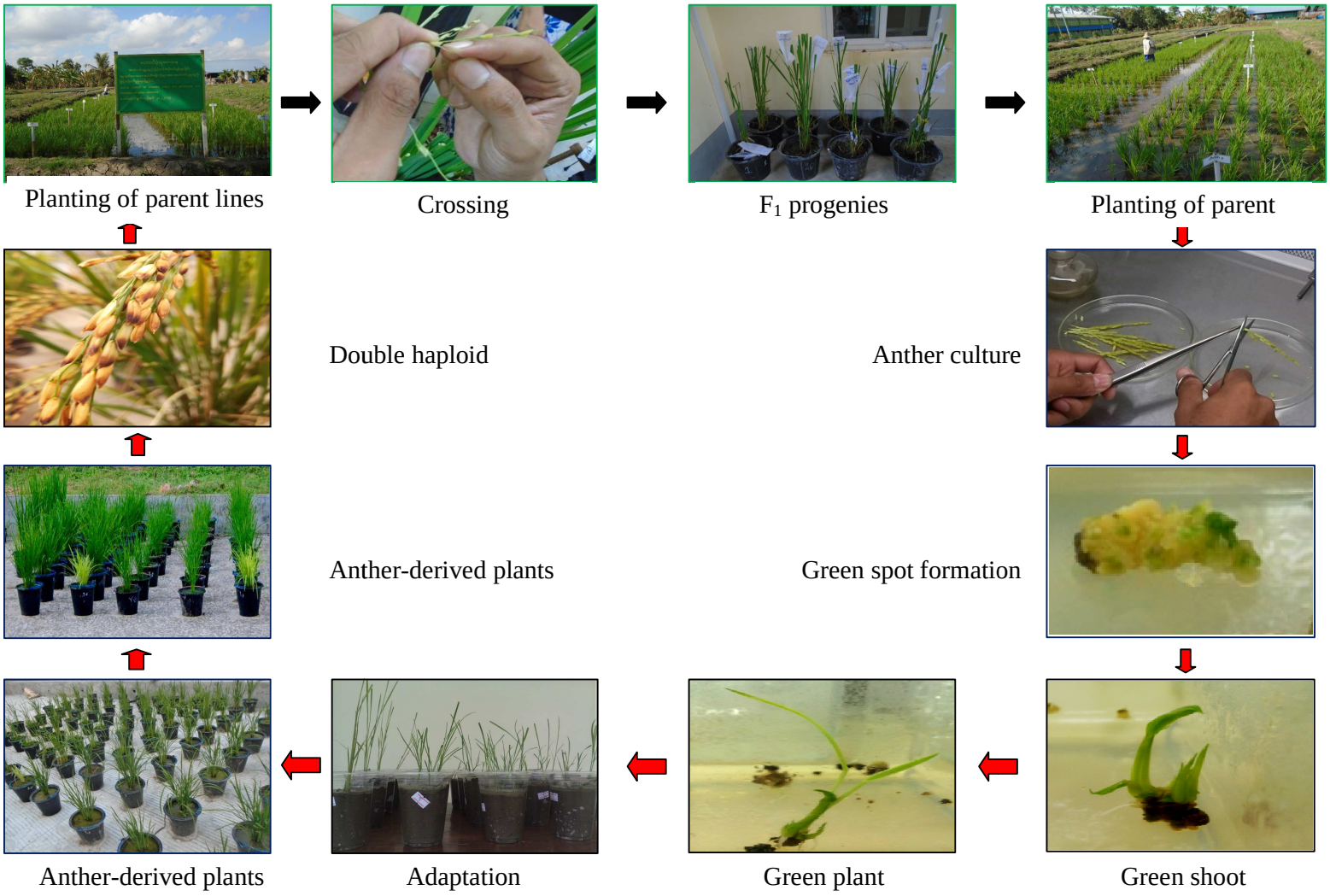

Figure 4.7 Protocol for androgenesis of rice

CHAPTER V
CONCLUSION

In this study, almost tested genotypes produced callus except one genotype (Sin Thwe Latt x Sin Akari-3) which showed no callus induction. The callus induction varied from 0.1-2.6 % depending on genotypes. The green plant regeneration depends on genotypes ranging from 6.3-37%.

There was strong genotypic effect on callus induction and plant regeneration. Among parents, the highest callus induction was observed in Yar-8, and Thee Htet Yin and Sin Thwe Latt were the lowest callus induction. Yar-8 and Shwe Man-1 showed the best callus induction and green plant regeneration, and the crosses involving these genotypes as femal parents were more responsive than others in callus induction. The cross containing Yar-8 as female with Thee Htat Yin, produced the highest green plant regeneration among crosses. The green plant production was also found in Shwe Man-1, Yar-8 and Yar-8 x Thee Htat Yin genotypes.

The anther-derived plants of these three genotypes were haploid and double haploid plants by panicle performance. Haploid plants can be treated with colchicines solution to obtain homozygous double haploid lines. Therefore, Yar-8 genotype has the potential to produce double haploid lines and it should be selected a suitable genotype for double haploid production in rice breeding program in Myanmar.

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APPENDICES

Appendix-1 Selected Indica Rice Genotypes

No.	Genotypes	Callusing Response	Characteristics	Life Span
1	Shwe Man -1	High	Drought Tolerance	125 -130
2	Sin Thwe Latt	Medium	Salt tolerance	135 -140
3	Sin Akari-3	No Response	High yield	130 -135
4	Thee Htat Yin	High	Quality rice	115 -120
5	Yar-8	Medium	Drought tolerance	120 -130

Appendix-2

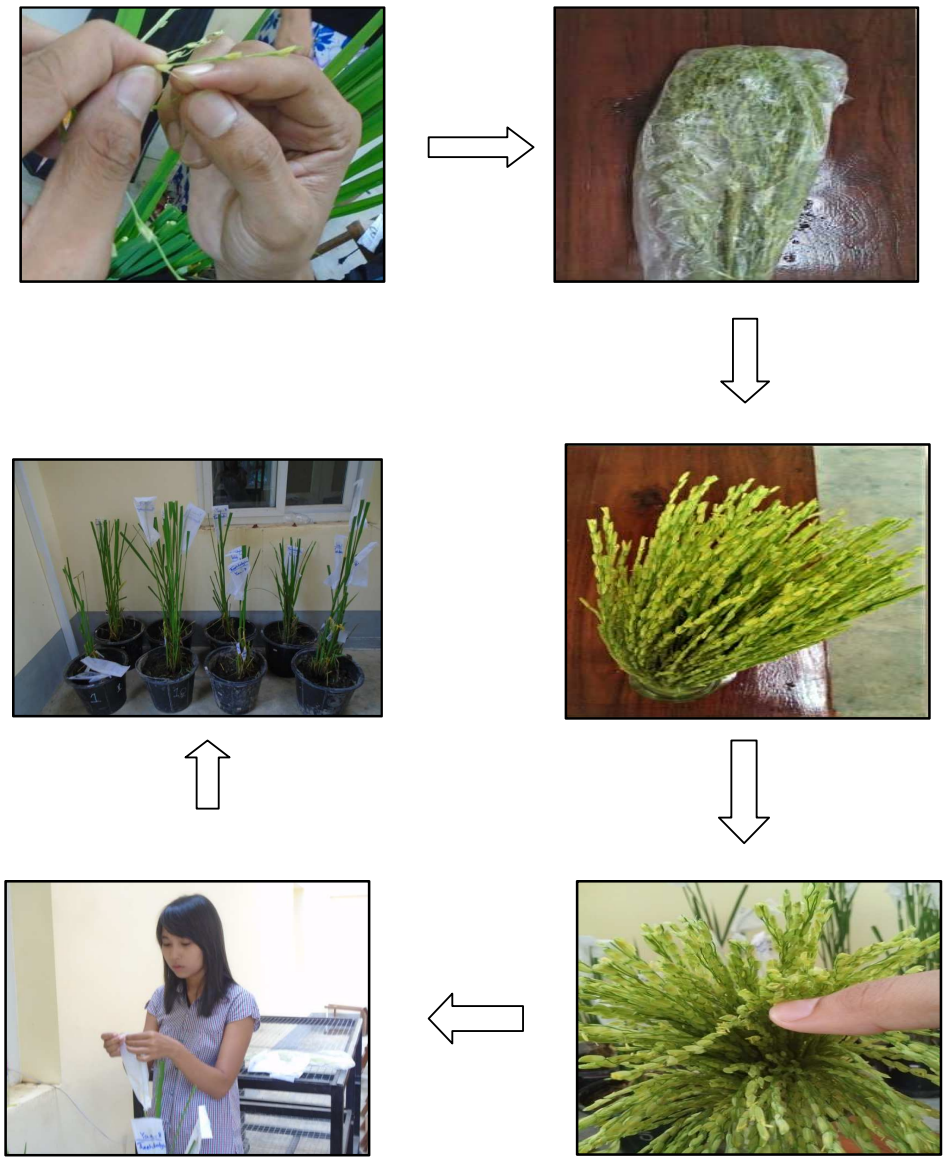


Plate 1. Steps for Hybridization

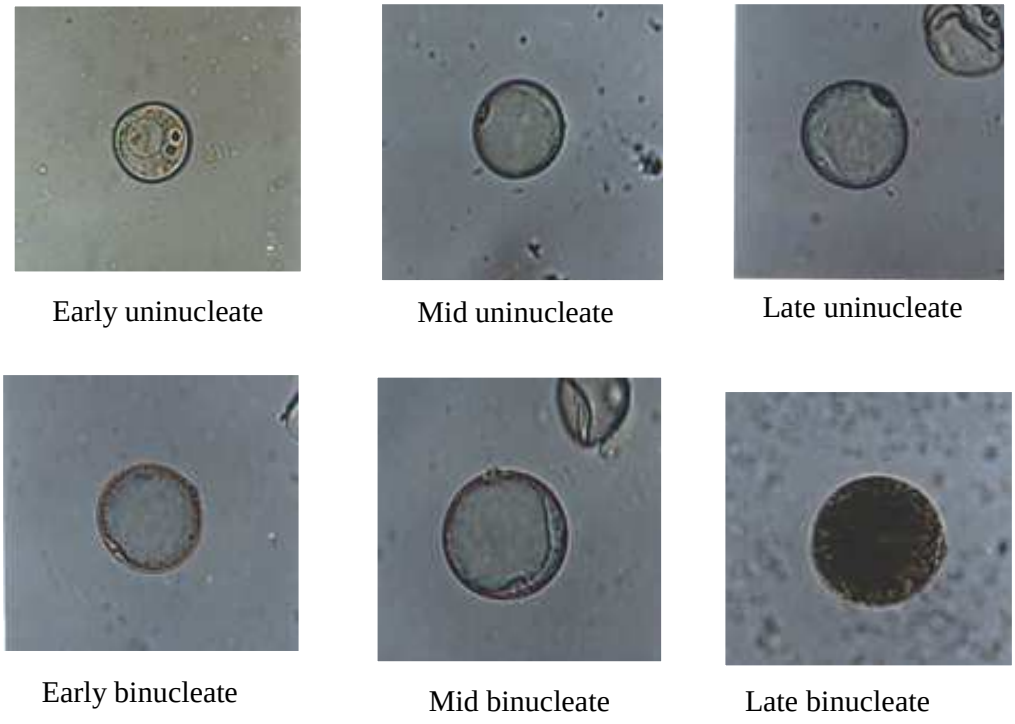
Appendix-3 Chemical composition of N_6 and MS media

Constituents (mg/l)	N_6	MS
Macronutrients		
KNO_3	2830	1900
KH_2PO_4	400	170
$(NH_4)_2SO_4$	463	1650
$MgS0_4.7H_2O$	185	370
$CaCl_2.2H_2O$	166	440
NH_4NO_3	-	1650
Micronutrients		
KI	0.8	0.83
H_3BO_3	1.6	6.2
$MnSO_4.4H_2O$	4.4	16.9
$ZnSO_4.7H_2O$	1.5	8.6
$Na_2MoO_4.2H_2O$	-	0.25
$CuSO_4.5H_2O$	-	0.025
$CoCl_2.6H_2O$	-	0.025
$FeSO_4.7H_2O$	27.8	27.84
$Na_2EDTA.2H_2O$	37.3	37.24
Vitamins		
Nicotinic acid	0.5	0.5
Pyridoxine HCl	0.5	0.5
Thiamine HCl	1.0	0.5
Glycine	-	2.0

Appendex-4 Chemical composition of Nutrient Yoshida Solution

Constituents	Amount (g/L)
$NH_4\ NO_3$	91.4
$NaH_2PO_4H_2O$	40.3
K_2SO_4	71.4
$CaCL_2$	88.6
$MgSO_4.7H_2O$	324
$MnCL_2.4H_2O$	1.5
$(NH_4)_6\ Mo_7O_{24}.4H_2O$	0.07
H_3BO_3	0.9
$ZnSO_4.7H_2O$	0.04
$CuSO_4.5H_2O$	0.03
$FeCL_2.6H_2O$	7.7
$C_6H_8O_7.\ H_2O$	11.9
$1MH_2SO_4$	50

Appendix-5



Early uninucleate | Mid uninucleate | Late uninucleate

Early binucleate | Mid binucleate | Late binucleate

Plate 2 Gametophytic pathway of microspore