IN VITRO REGENERATION OF SELECTED RICE (*Oryza sativa* L.) GENOTYPES THROUGH ANTHER CULTURE

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IN VITRO REGENERATION OF SELECTEDRICE (*Oryza sativa* L.) GENOTYPES THROUGH ANTHER CULTURE

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The thesis attached hereto, entitle "**In Vitro Regeneration of Selected Rice (*Oryza sativa* L.) Genotypes through Anther Culture**" was prepared under the direction of chairperson of the Candidate's Supervisory Committee and has been approved by all members of that committee as a requirement 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 author, except where otherwise stated. It has not been submitted previously for a degree at any University.

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DEDICATED TO MY BELOVED PARENTS,

U MYA SOE AND DAW SEIN WIN

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ABSTRACT

Rice is one of the most important cereal crops in the world. Anther culture, one of double haploid techniques, is simplest and most efficient technique for rice breeding. The present study was carried out to evaluate callus induction ability of selected rice genotypes on media supplemented with two different carbon sources and to investigate suitable BAP concentrations on plant regeneration of anther derived callus. The selected 19 rice genotypes (14 indica and 5 tropical japonica) were used in the experiment. Anthers of each genotype were cultured on N6 medium supplemented with two types of carbon source (4% maltose or 4% sucrose), 2mg.L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) and 0.5 mg.L^{1} kinetin for callus induction. Anther derived callus was transferred to Murashige and Skoog (MS) medium supplemented with (0, 0.5, 1) mg.L^{-1} 6-Benzylaminopurine (BAP), 1mg.L^{-1}1-Naphthaleneacetic acid (NAA) and 1mg.L^{-1} l kinetin for regeneration.

Seventeen out of 19 rice genotypes produced callus on media supplemented with maltose while only 13 genotypes produced callus on media supplemented with sucrose due to genotype dependency. Tropical japonica was more responsive to induce callus from anther than indica among the tested genotypes. Callus induction varied from 0.44% to 19.22%. Tropical japonica (Paw San Taung Pyan Hmwe) has highest callus induction (19.22%) than the other tested genotypes. Yebaw Sein has highest callus induction (6.78%) among the tested indica rice genotypes. Plant regeneration from callus varied from 0% to 25.15%. In tropical japonica genotypes, Bay Kyar Taung Pyan was recorded maximum green plants regeneration (25.15%), while Hnan Kar was (13.33%) among indica genotypes. In this experiment, both MS media supplemented with 0.5mg.L^{-1} BAP and 1mg.L^{-1} BAP showed maximum green plants regeneration although maximum green spots formation occurred only on 1mg.L^{-1} BAP. Maximum green plant was found in Bay Kyar Taung Pyan in tropical japonica and Lat Yone Kyi in indica rice.

Key words; anther culture, rice genotypes, carbon sources, plant growth regulators, callus

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CHAPTER I
INTRODUCTION

Rice is one of the most important cereal crops in the world because it has a more complex nutritional content than other cereals, including proteins, carbohydrates, vitamins and minerals. About 85% of the total world rice production is produced and consumed in Asian countries. The original habitat of rice is found to be plains along the Himalayan foot - hills, the central Myanmar and Thailand. Myanmar is also recognized as an original habitat of rice (MOAI 2004).

Being an important crop for many Asian countries, rice (*Oryza sativa* L.) is grown in various ecosystems of all over the country. The total cultivated area of rice in Myanmar was 7.17 million hectares of which 6.45 million hectares are monsoon rice and 1.18 million hectares are summer rice in 2014-2015. The total rice production was 28.19 million metric tons with the average yield of 3.94 Mt ha^{-1} in Myanmar (MOAI 2015).

There have been some problems concerned with food security throughout the world. The global population will be 8.42 billion in year 2025. Rice consumers are increasing at the rate of 1.8% every year. The rate of growth in rice production has slowed down due to environmental degradation. It is necessary to increase rice production (Mishra et al. 2015).

Many plant breeders have been used conventional methods to get elite variety of rice. In conventional methods, the varietal improvement was done by selecting individual within a population. It is time consuming process to produce a new variety. Advanced technologies to complement conventional plant breeding techniques must be employed to cope with the increasing demand to produce high yield, high protein and disease resistance rice (Soe 1988).Tissue culture techniques such as anther culture, embryo rescue, somatic hybridization and use of somaclonal variation have contributed to the release of new rice variety (Sammour et al. 2015).

Among tissue culture techniques, double haploid technique which accelerate the breeding cycle and allow better discrimination between genotype within a generation became very attractive. Anther culture, one of double haploid techniques, is simplest and most efficient technique for rice breeding. The production of doubled haploids through anther or isolate microspores culture *in vitro* is a rapid approach to homozygosity that shortens the time required for development of new rice cultivars

through conventional methods, which require at least 6-7 generations (Gueye and Ndir 2010).

There are three types of rice, javanica, japonica, and indica. Most of rice varieties grown in Myanmar are indica varieties and some japonica rice is rarely grown (Aung et al. 2009). A large numbers of good quality rice genotypes are being indigenous to Myanmar. Although some efforts have been made for rice anther culture, it is necessary to improve the research activities for remaining rice genotypes.

Callus induction of indica cultivars was found to be extremely poor compared to japonica cultivars (Grewal et al. 2011). In the case of indica rice, early anther necrosis, poor callus proliferation and albino plant regeneration are currently recognized as the major problems (Jha 2016). Many researchers reported that different rice species, subspecies or genotypes behaved differently in response to anther culture, and genotype is the main important factor for the success of anther culture (Niroula and Bimb 2009).

Choice of carbohydrate used as carbon source in the medium may play an important role in callus induction. Sucrose is most commonly used carbohydrate source for all types of tissue cultures. Maltose has been known as a better carbohydrate source in comparison to sucrose for androgenesis in many plant species (Bagheri et al. 2009).The effect of maltose and sucrose on androgenesis is variety specific (Swapna 2000).

The success of plantlet regeneration under *in vitro* culture system depends on type and dose of different growth regulators especially auxin and cytokinin used in cereal anther culture (Roy and Mandal 2005). A number of experiments showed that using hormone combinations and ratios gained the good induction effects. High concentrations of auxin lower the ability of callus to produce green plant. High level of auxin addition with kinetin at low concentration encourages callus induction and subsequent plantlet regeneration (Herath et al. 2008).Callus formation and plantlet regeneration were both stimulated with the use of kinetin. Combination of kinetin, NAA and IAA obtained a higher rate of regeneration (Chaleff and Stolarz 1982). BAP was the most effective cytokinin in enhancing plant regeneration efficiency (Htwe et al. 2011).

In this regard, the experiment was conducted with the following objectives -.

- To evaluate callus induction ability of selected rice genotypes on media supplemented with two different carbon sources
- To investigate suitable BAP concentrations on plant regeneration of anther derived callus

CHAPTER II
LITERATURE REVIEW

2.1 Early History of Anther Culture

The existence of only one set of chromosomes in their cells is recognized as haploid plant. In nature, haploids occur as an irregularity when the haploid egg forms an embryo without fertilization. Chromosomes doubling are required to produce fertile plants which are called double haploids or homozygous diploids because haploids are sexually sterile (Bhojwani and Dantu 2010).

Doubled haploid production is a valuable technology required for F1 hybrid breeding. The discovery of doubled haploidy has changed breeding programs of many globally important crops and nowadays mainly based on the use of this innovative and efficient technology (Forster et al. 2007). Over 20 rice varieties have been reported through double haploid approaches in China, Korea, Japan and USA (Zepata-Arias 2003).

Double haploid plants can be obtained by several methods including isolated microspore culture, anther culture, wide hybridization and ovule culture and choice of method varies upon crops to develop haploid (Asif 2013a)

Since 1964, the technique of anther culture was first developed in *Datura* by Guha and Maheshwari. Anther culture is an innovative method for hastening the generation of homozygous double haploid (DH) and can be used to accelerate the varietal improvement programs (Herawati et al. 2010). Several scientists have successfully produced callus and haploid plants through anther culture and isolated pollen and anther (Bhojwani and Dantu 2010). In 1985, double haploids have been produced via anther culture in over 170 species. Success of anther culture methodology has been achieved in crop species such as triticale, wheat, rice, barley, rye, and several others like medicinal, vegetables, fruits, ornamental, and woody plants (Dunwell 2010).

In 1968, anther culture was applied in rice to produce haploid plants by Niizeki and Oono. Although anther culture is recognized as a valuable tool in plant breeding programs, various factors such as high genotypic dependency and low frequencies of callus induction and plant regeneration limit its actual application (Medhabati et al. 2014). Success of anther culture in rice has been influenced by numerous endogenous and exogenous factors such as genotypes, developmental stage

of microspore, cold treatment of anthers, culture medium and the choice of plant growth regulator (Asif 2013b).

2.2 Factors Affecting on Anther Culture

2.2.1 Genotypes

Genotype is the main factor for anthers culture process. Genotypes of material used may be the first important factor which affects the productivity in rice anther culture (Win 1994). It has been suggested that in rice, callus induction and green plant regeneration were mainly controlled by additive genetic effects (Yan et al. 1996).

2.2.1.1 Genotypes effect on callus formation

In most of the species, genotype is a deciding factor as different rice species, subspecies and varieties behave differently in their response (Ramakrishnan et al. 2005). The callus induction depending on the different genotypes in the same medium was reported by Mu (1999). The results of Myint (2002) pointed that with all other culture conditions being constant, different genotypes respond differentially to callus induction and finally to plant production.

Aung et al. (2009) reported that 43 out of 55 indica rice genotypes were initiated callus while 12 genotypes did not in their study. Herath and Bandara (2011) reported that the frequency of anther forming calli varied from 6.07% to 15.58% depending upon the genotypes used in their study.

Out of 17 tested genotypes evaluated, 12 responded in N6 medium and callus induction frequency varies from 0.2 to 29.4% (Herath et al. 2007). Callus induction commenced three weeks from culture and the frequency of the anthers forming calli varied between 4.3% and 78.3% depending upon the genotypes (Herath and Bandara 2011).

Kaushal et al. (2014) reported that the genotypic difference for anther culture response varied from 6.07% to 15.58% was observed among the tested 13 genotypes. This indicates that genotype is the deciding factor for anther culture.

Even among different genotypes of a particular ecotype, indica or japonica, considerable variation in pollen callusing and green plant regeneration has been observed with the genotypic effect being greater among the indica types (Silva 2010). Kaushal et al. (2014) reported that anther culture response varied from 42% for a japonica cultivar to 0% for an indica cultivar. The response to anther culture in rice, in terms of frequency of callus induction and plant regeneration, is highest in

japonica/japonica and followed by japonica, indica/japonica, indica/indica, indica respectively (Kaushal et al. 2014).

2.2.1.2 Genotypes effect on plant regeneration

Plantlet regeneration is a genotype specific character (Abyawickrama and Anai 2006). Callus induction and plantlet regeneration were found more in the japonica varieties than the best indica varieties. Frequency of green plant regeneration initiated from calli varied between 2.2 to 69.7 % depending upon the genotypes (Herath and Bandara 2011).

Aung et al. (2015) tested anther culture capacity of 12 tropical japonica genotypes. They found that only three of tested genotypes produced green plant although all genotypes induced calli. Ranjana et al. (1998) observed that green plant regeneration frequency range from 0 to 26.86% in indica cultivars (Basmati 370, Tulsi and Tetep), depending upon the genotype and the constituents of the media used. Dewi et al. (2009) reported that plant regeneration frequencies of 10 % from anther culture are in common in japonica, efficiencies of 3% are considered high for indica.

Several factors, including pre-treatment, culture medium and the protocol, affect the frequency of albinos. Albinism may be one of the expressions of genetic deficiency. Indica rice cultivars are more prone to this problem than japonica rice (Raina et al. 2001).The frequency of albinos may vary from 5 % to 100 % (Talebi et al. 2007).

The literature on androgenesis in cereals suggests that albinism can be considerably reduced by shortening the culture period (Asaduzzaman et al. 2003). Torp and Andersen (2009) founded that combination of starvation and cold stresses applied simultaneously for shorter periods (3–4 days) have increased microspore survival and reduced the frequency of albino production compared to only prolonged cold pretreatments.

2.2.2 Developmental stage of pollen

The developmental stage of microspore at the time of anther excision and plating for *in vitro* culturing is also an important factor in androgenesis (Seguí-Simarro and Nuez 2008). In nearly all responsive crops, including model species such as rapeseed and tobacco, the inducible stage revolves around the first pollen mitosis, from vacuolate microspore to early bicellular pollen (Touraev et al. 2001). For species cultured during the uninucleate stage, the microspore either undergoes a normal

mitosis and forms a vegetative and a generative nucleus or divides to form two similar looking nuclei. In those cases where a vegetative and a generative nuclei are formed in culture, or where binucleate microspores are placed into culture, it is usually the vegetative nucleus that participates in androgenesis (Salvia 2010).

The enhancement of anther culture efficiency has been contributed by correctly identifying the pollen stage of anthers in rice varieties (Silva and Ratnayake 2009). In rice, anthers in which microspores are at the uninucleate stage are the most responsive and suitable for culture (Mishra and Goswami 2014). The response of anthers at the tetrad stage is not good at all, and it falls sharply after the first pollen mitosis. At this stage, starch deposition begins, but no sporophytic development and subsequently no macroscopic structures form in the microspores. However, pollen at this stage, when cold pre-treated, can deviate from normal developmental programme and switch to sporophytic growth. Determining the developmental stage of pollen in the anthers is important to optimize the anther culture response of a given rice genotype (Silva 2010).

Genovesi and Magill (1979) stated that uninucleate pollens stage were effective in culture of rice. About 90% of plants regenerated from uninucleate microspores were green although binucleate stage microspore produced more albino plants than uninucleate stage.

If the uninucleate stage was further divided into three sub stages, the early-, mid- and late- uninucleate stages, the peak response occurred at the mid-uninucleate stage. The response declined sharply before or after mid-uninucleate stage. The frequency of callus induction of three uninucleate stage, are 5.6%, 35.7%, 10.5% in early, mid- and late-nucleate stage respectively (Chen et al. 1997). There has been a general agreement in the literature that the uninucleate stage of microspore development is the most desirable for rice anther culture (Win 1994).

Exact determination of pollen stage requires a cytological analysis but for practical large-scale programme many investigators prefer to rely on a simply measured, although less reliable, external morphological indicator, such as corolla length (Dunwell 2010). Bishnoi et al. (2000) reported that the distance from flag leaf to penultimate leaf auricle can be a convenient morphological marker for estimating the maturity stage of pollen in different rice varieties. Early anther culture research papers also indicated that panicle selection for anther culture could be based on the extension of flag leaf sheath, determined by measuring the distance the flag leaf collar

and the collar of the penultimate leaf. Panicles at the booting stage, 7–10 cm in length, that were positioned between the subtending leaf and the flag leaf were collected. During this period, the anther sac frequently develops to the middle and late uninucleate cell division stage (Afza et al. 2000).

2.2.3 Cold treatment

Various types of pretreatments such as cold and heat shocks, starvations in the form of nitrogen and carbohydrates, irradiation or chemical treatments were used in anther culture to induce stresses that can ultimately help to switch gametophytic pathway of microspore to sporophytic development (Asif 2013a). Certain treatments given to the whole spike or to the anther have an effect on the development of the microspore. The most commonly used in rice anther culture is cold shock treatment (Kaushal et al. 2014).

Some of the positive effects of cold pretreatment include delay of anther wall senescence, increase of symmetric divisions of pollen grains and metabolic changes necessary for androgenesis (Kaushal et al. 2014). As the result of cold shock, weak or non viable anthers and microspores are killed, they then become dark brown in color. Thus, the spikelets are enriched in vigorous anthers. At low temperature increased number of microspore are arrested during the first mitosis, as starch production is blocked. This increases the possibility of subsequent development. It is also possible that the cold treatment retard aging of anther wall, allowing a higher proportion of microspores to change their developmental pattern from gametophytic to sporophytic and guide the continuous division of the microspore to form callus (Mishra and Rao 2016).

There was no callus development observed in the anthers without cold pre treatment (Herath et al. 2009). Low temperature shock enhances the androgenic response in several species including rice (Sen et al. 2011). Cold treatment is essential for improved anther culture response, and manipulation of pretreatment has the ability to improve the callus induction and subsequent green plant regeneration (Kaushal et al. 2014).

Indica and japonica varieties are known to differ in requirements for cold treatment (Kaushal et al. 2014). The temperature and duration of the cold pretreatment varies with the genotypes. In rice, different temperatures associated with incubation time were found to give better results. It indicates that, each genotype requires

specific pretreatment temperature and incubation period for optimum callus response (Kiviharju and Pehu 1998).

In 1989, Pathinayake and Johnsion reported that a possibility of increasing the callus induction of cultured anthers significantly by a constant temperature treatment at 12ºC for 8 days for both japonica and indica genotypes.

Kaushal et al. (2014) reported that the best performance for callus induction and plant regeneration was observed when the anther was cold treated at 12º C for 5 days.

The highest callus induction (88.88%) and efficiency of green plant regeneration (24.25%) of IR 43 were found at 5 days incubation periods at 4-7ºC (Khatun et al. 2012). Cold pre-treatment at 8ºC for 14 days gave the best performance in callus induction and plant regeneration in selected Indica and Japonica parents and their F1 hybrids (Herath et al. 2009).

Pande (1997) observed that cold pre-treatment was essential for androgenesis in anther cultures of the indica cv IR43, and 10ºC for 10 days was the most suitable. Zapata-Arias (2003) reported that temperatures from 8 to 10ºC for 8 days have been recommended to be optimal for many varieties of rice.

The time needed for pretreatment depends on the temperature used (Win 1994). In general lower the temperature (as low as 3-5ºC) the shorter the time is needed and the higher (as high as 10-15ºC) the longer (Myint 2002). Although a cold shock for 25 days enhanced the frequency of callusing in rice, the plantlets regenerated from these microspores were mostly albinos (Herath et al. 2009).

Prolonged pretreatment over optimum was proved to be inhibitory and has an adverse effect on green plant formation and significant increase in albino incidence (Lenka and Reddy 1994). Albino formation also followed a pattern in relation to the duration of cold shock (Kaushal et al. 2014). Cold treatment shift from gametophytic mode of development to sporophytic mode may cause instability and the loss of chlorophyll is a manifestation of that shift. Although albinism is genetically determined, manipulation of pretreatment duration can reduce the frequency to some extent (Rukmini et al. 2013).

2.2.4 Culture medium

Rice anther culture is a two-step process; initial development of callus and subsequent regeneration of green plants from embryogenic callus. The nutrient

requirements of the two processes vary and therefore are facilitated on different culture media (Silva 2009).

Nutritional requirements for optimal growth of a tissue *in vitro* may vary with the species. The influence of media composition has been one of the most importances in anther culture. However, there is no general agreement on which media are superior as variety and medium interactions are almost significant. There is no universal medium, in which anthers from all varieties are capable of high percentage callus induction. The induction frequency for the same genotype varies greatly with the composition of medium. Thus, the function of medium is not negligible (Win 1994).

The choice of medium has made a difference in the rate of success in callus induction ability of japonica and indica genotypes (Silva 2009). The most widely used medium for inducing higher anther response in japonica cultivars is the N6 medium although the basal nutrients of this medium are not optimum for anther culture of indica rice (Lentini et al. 1995).

The He2 medium was recorded to be better than N6 medium in the study of 8 indica cultivars. He2 medium is derived from N6 medium by reducing NH_4^+ to half strength and $MgSO_4$ to $1/50^{th}$ level, and doubling the concentration of KH_2PO_4 (Kikubhai 2010). Based on detailed study on the medium requirement of the indica cultivar IR43 evolved a new medium called M-019 (Raina and Zapata 1997). Oono (1975) examined several other media and recommended MS media as the most suitable medium for anther culture. Subsequent developments have focused mostly on affecting modifications to the two basic media, N6 and MS, for improving anther culture efficiency in indica rice. Several modifications to the basal N6 medium have been tested for their usefulness for anther culture of indica rice (Silva 2009).

2.2.4.1 Nitrogen sources

Supply of nitrogen in a specific form plays an important role in *in vitro* culture of rice explants. Thus it is advisable to supply nitrogen in the form of ammonium rather than nitrate. A concentration of 3.4mM ammonium in the culture medium gave better response for indica rice anther cultures (Kikubhai 2010). In 1975, Chu demonstrated that the level of ammonium nitrogen in the culture medium is critical for androgenesis in rice. In tissue culture media, inorganic nitrogen is usually supplied in the form of nitrate and/or ammonium ions (Silva 2009).

Mixture of ammonium and nitrate is the most suitable inorganic nitrogen source for growing plant cells (Yatazawa and Furuhash 1968).The development of the pollen callus require both KNO_3 and $(NH_4)_2SO_4$ but a high $(NH_4)_2SO_4$ concentration would inhibit the formation and growth of pollen callus(Bishnoi et al. 2000).Nitrogen supplied only in the form of nitrate or ammonium ions in the medium was less beneficial for induction of morphogenic calli from cultured anthers than a combination of both at appropriate concentrations (Raina and Zapata 1997).The medium containing $(NH_4)_2SO_4$ became acidic soon after the culture was incubated, and there was hardly any growth of the callus. With KNO_3 as nitrogen source, callus became necrotic at healthy regions of the tissue after about seven days. This adverse effect was due to the formation of nitrite from nitrate during the growth of the callus (Yatazawa and Furuhash 1968).

The N6 medium contains both KNO_3 (28 mM) and $(NH_4)_2SO_4$ (3.5 mM) as primary sources of nitrogen and used successfully for japonica varieties. The ratio of NO_3^-:NH_4^+ has been observed to be an important determinant for the success of anther culture in indica rice (Kaushal et al. 2014). Ogawa et al. (1995) studied the effect of nitrogen source on androgenesis in indica cultivar IR24, using R-2 medium as the control. R-2 has 40 mM KNO_3 and 2.5 mM $(NH_4)_2SO_4$. When 20 mM KNO_3 was combined with a small amount of $(NH_4)_2SO_4$, glutamine or alanine all treatments induced pollen callusing but alanine was the best supplement. It not only induced high frequency androgenesis but also showed maximum regeneration of green plants.

Nitrogen may also be added in an organic form as amino acids. Amino acids such as glutamine and alanine have proved to be useful for callus formation and green plant regeneration from microspore culture in indica rice varieties (Ogawa et al.1995). Proline and glutamine promoted callus formation in microspore cultures of a japonica cultivar. These amino acids also induced a higher degree of plant regeneration and green plant production than the medium containing no amino acid. However, organic form of nitrogen cannot totally replace inorganic forms (Haque et al. 2015).

Calcium in the medium is known to stimulate ethylene production in many plant tissues. Addition of the calcium ion ophore A23187 (0.5 mM) along with $CaCl_2$ (1 mM) enhanced pollen callusing over $CaCl_2$ alone. Addition of 10 mg L^{-1} of $AgNO_3$, an anti-ethylene compound, to the callus induction medium enhanced pollen callusing frequency in indica cultivars from 10.1 % to 20.6 %. With $AgNO_3$ the frequency of green plant differentiation doubled (Bhojwani and Dantu 2010). The

addition of $AgNO_3$ to the medium improves callus induction and plant regeneration in indica rice (Faruq et al. 2014).

2.2.4.2 Carbon sources

It has been known that some carbohydrate source, sucrose, is most commonly used for all types of tissue cultures. The kind, type and concentration of carbohydrate used in the medium for inducing *in vitro* androgenesis varies from species to species (Mishra and Goswami 2014).

The requirement of sucrose for successful androgenesis was first demonstrated by Nitsch and Nitsch in 1969 for tobacco and later by Sunderland et al. (1974) for *Datura innoxia* .In early studies, media designed for rice anther culture used sucrose as the standard carbon source (Silva 2009). The effect of sucrose on anther culture has been applied in a number of species. Sucrose concentration in induction medium has a major effect on osmosis and the development of embryos is apparently influenced by osmosis. The normal level of sucrose is 2-4%. High sucrose concentration favors better survival of pollen grains (Mishra and Goswami 2014).

Specific modification of the carbon source includes the disaccharide maltose in species including potato, barley, lupin, pepper and oat (Dunwell 2010). Since 1983, maltose has been shown to be a superior source of carbohydrate than sucrose for androgenesis in several species, including cereals (Pande and Bhojwani 1999). In anther culture response, media incorporated with different sugars such as glucose, fructose, mannitol or a combination of these at concentrations that produce equivalent osmotic effects in the medium, have proven to be much less effective than maltose (Bishnoi et al. 2000).

Replacing sucrose (146 mM) with maltose (146 mM) in the callus induction medium had a significant positive effect on anther response in both indica and japonica types. Maltose enhanced pollen callusing from 6.3 % to 10.1 % and green plant regeneration from 0.6 % to 1% (Silva 2009). Javed et al. (2007) reported that maltose gave better results than sucrose at the callus induction stage and resulted maltose effect is greater 1.5 times than sucrose for callus productivity of tested cultivars. Maltose has been reported to be a superior source of carbohydrate than sucrose for androgenesis in several species, including cereals (Sen et al. 2011).

Maltose in the anther culture medium is degraded more slowly than sucrose and yields only glucose upon hydrolysis. Sucrose is metabolized very rapidly into glucose and fructose. Fructose is known to have a detrimental effect on embryoid

production in wheat anther culture and may also be the reason why maltose supports androgenesis better in rice than sucrose (Bagheri et al. 2009).

2.2.5 Plant growth regulators

In addition to the nutrients, it is generally to add one or more growth substances, such as auxins, cytokinins and gibberellins, to support good growth of tissues and organs. The requirement for the substances varies considerably with the tissue (Bhojwani and Razdan 1983). For rice anther culture, auxins and cytokinins are the only organic growth factors required as growth regulators (Win 1994).

2.2.5.1 Auxin

The auxins commonly used in tissue culture are IBA (indole-3-butyric acid), NAA (naphthaleneacetic acid), NOA (napthoxyacetic acid), PCPA (parachloro-phenoxy acetic acid), 2,4-D (dichloro phenoxyacetic acid) and 2,4,5-T (trichloro - phenoxy acetic acid). Auxins were essential for the induction of callus, and type and concentration of auxins also influenced this process (Kaushal et al. 2015).

Auxin commonly used in the callus induction medium for rice anther culture is 2,4-dichlorophenoxyacetic acid (2,4-D). Different concentration of 2,4-D was most widely used in MS medium for sufficient callus induction and 2mg.$L^{-1}$was suitable (Silva 2009).

2, 4-D has proven to be an effective auxin for callus induction from cultured anthers. It promotes rapid cell proliferation and formation of non-embryogenic callus and has been accepted that 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 (Raina and Zapata 1997).

NAA is also one of the most commonly used auxins required for induction of callus from anthers of cereals. Calli formed in the presence of NAA were more capable of plant regeneration than those initiated in media with 2,4-D (Zhu et al. 1998).

2, 4-D inhibits the organogenesis of calli, and NAA promotes the formation of roots and sometimes completes plants (Mishra and Rao 2016). Neither 2,4-D nor NAA can support regeneration, and the use of cytokinins like kinetin and benzyl amino purine are required (Mandal and Gupta 1995).

Increased auxin concentration could be beneficial for callus induction, but detrimental for green plant regeneration. There appeared to be a relationship between

increased albino developments with increase in level of auxins. Genotype, Medium, Auxin and Cytokinin synergistically contribute to enhanced callus induction and regeneration of androgenic plants (Kaushal et al. 2015).

2.2.5.2 Cytokinin

Cytokinins added to the medium used are very important during tissue culture of plants because they induce division and organogenesis (Howell et al. 2003) and affect other physiological and developmental processes (Van Staden et al. 2008). Cytokinins most often used in tissue cultures are BA, Kin and 2iP as well as thidiazuron. The effectiveness of BA to stimulate growth of axillary shoots *in vitro* is well described (Nobre et al. 2000). High concentration of BA may cause shoots vitrification (Huang et al. 1998).

Among cytokinins, kinetin, 6-benzylaminopurine (BAP), and zeatin (Z) have been frequently tested both in callus induction and regeneration media (Dubas et al. 2015).

Kim et al. (1992) reported that in the presence of NAA at low concentration and 2-10 $mg.L^{-1}$ kinetin, green calli were induced from excised embryos. They also observed that chlorophyll content increased as kinetin concentrations increased however callus induction decreased.

2.2.5.3 Ratio of auxin and cytokinin

Both auxin and cytokinin are crucial constituents in rice anther culture medium, control the dedifferentiation and dedifferentiation processes in the *in vitro* cultures (Kaushal et al.2015).

Combination of cytokinin and auxin is recognized to promote plant regeneration frequency in some rice cultivar (Rueb et al. 1994). 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).

Combination of lower level of 2,4-D (0.5mg.L-1) and NAA (2.5mg.L-1), and kinetin (0.5mg.L-1) have been used effectively to induce callus from several indica varieties (Shahnewaz and Bari 2004).

Rukmini et al. (2013) reported the different auxins (2, 4-D, NAA) and cytokinins (Kinetin, BAP) and their combinations studied. A ratio of 1:4 for 2,4-D

and NAA and 1:3:1 ratio of Kinetin: BAP: NAA ratio proved to be optimal for callus induction and green plant regeneration respectively.

Growth regulators used in the regeneration medium and their effects on green plant production has been analyzed to a lesser extent than in the case of callus induction (Silva 2009). Bishnoi et al. (2000) reported that medium supplemented with NAA (0.5 $mg.L^{-1}$), BAP (1.0 $mg.L^{-1}$) and kinetin (1.0 $mg.L^{-1}$) has adequately supported green plant regeneration from sub-cultured callus.

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Genotypes

Nineteen rice genotypes (Table 3.1) were selected and these were provided by Seed Bank and Rice Division, Department of Agricultural Research (DAR), Yezin.

Table 3.1 Nineteen selected rice genotypes used in the experiment

No.	Genotypes	Types	Life Span
1	Yar-8	Indica	120-125
2	Thee Htat Yin		115-120
3	Shwe Pyi Htay		127
4	Thee Htat-3		125-130
5	Hmaw Bi 3		125-130
6	Hmaw Bi 2		140-145
7	Sin Thwe Lat		135
8	Thu Kha Hmwe		137
9	Kone Myint 2		140
10	Shwe War Htun		145
11	Lat Yone Kyi		Late Nov
12	Hnan Kar		Late Oct
13	Yebaw Sein		Mid Dec
14	Shwe Ta Soke		Early Nov
15	Paw San Hmwe(Acc No. 002500)	Tropical Japonica	Late Nov
16	Paw San Taung Pyan Hmwe (Acc No. 002924)		Mid Dec
17	Paw San Bay Kyar (Acc No.002925)		Late Nov
18	Bay Kyar Taug Pyan (Acc No.001208)		Mid Dec
19	Nga Kywe Taung Pyan (Acc No.002054)		Mid Dec

3.1.2 Culture media

Chu (N6) was used as callus induction medium and Murashige and Skoog (MS) was used as regeneration medium. Yoshida's nutrient solution was used as medium for root development.

Chemical components and amounts for each medium were described in appendix 1 and 2.

3.1.3 Carbon sources and plant growth regulators

Sucrose (40 $g.L^{-1}$) and maltose (40 $g.L^{-1}$) were used as carbon sources in callus induction stage. 2,4-Dichlorophenoxyacetic acid (2,4-D), and kinetin were used as plant growth regulators in callus induction stage. Plant growth regulators in plant regeneration stage were Kinetin, 1-Naphthyl Acetic Acid (NAA), and Benzylaminopurine (BAP).

Carbon sources and plant growth regulators used in the experiment were described in appendix 3.

3.2 Materials Preparation

3.2.1 Planting the seeds

Just before planting, seeds of each genotype were dried under the sunshine for 24 hours. Then, the seeds were soaked in water for 24 hours and incubated at room temperature for germination. The seeds were sown in the tray. Twenty days old seedlings were transplanted in the field of Department of Horticulture and Agricultural Biotechnology with regular supply of water and followed recommended cultural practices. All possible care was applied to protect plants from pests and disease infection.

3.2.2 Boots collection

Healthy panicles of each genotype were collected at the uninucleate stage of microspore development in anther. Nuclear stage of pollen was examined cytologically and panicle morphology was examined. According to plant morphology, uninucleate stage of microspore is that the flag leaf base distance from the first lower leaf is 7-10 cm for indica types and 10-15 cm for japonica types. At least 100 panicles were collected from each genotype.

3.2.3 Cytological examination

Stage of microspore development was determined by ‘Squashing’ the entire anthers in the solution of ethanol: chloroform: acetic acid in the ratio of 6:3:1 and kept in room temperature for 24 hours. Anthers were stained with potassium iodide and observed under a low power objective of light microscope (Chang et al. 2014). Boots with anthers that contained mid-uninucleate to early binucleate stage pollen were used in the experiment. Boots which contained very young or very old pollen stages were discarded.

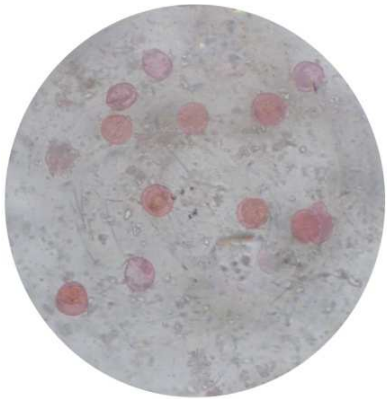

Plate 1 Cytological examination of anther under microscope

3.2.4 Pre-culture treatment or cold treatment

The collected boots were washed thoroughly with tap water and surface sterilized with 90% ethanol, wrapped with paper, covered with plastic and kept in refrigerator at 10°C about 8 to10 days for cold shock treatment.

3.3 Methods

3.3.1 Callus induction

3.3.1.1 Sterilization of boots

Cold treated boots were surface sterilized with 70% ethanol for 3 minutes and in sodium hypochloride (2%) solution for 20 minutes and followed by rinsing three times thoroughly with distilled water. This sterilization step was done under aseptic condition.

3.3.1.2 Anther incubation

Anthers with right developmental stage were cultured into 25×150 culture test tube containing 15 ml of callus induction medium.

3.3.1.3 Culture media

N6 solid media supplemented with 2 $mg.L^{-1}$ 2,4-D, 0.5 $mg.L^{-1}$ kinetin and two types of carbon sources (sucrose, maltose) were used for callus induction.

3.3.1.4 Culture condition

The culture test tubes were kept at 25±1°C under dark condition for callus induction.

3.3.1.5 Experimental Layout

The experiment was conducted using a two factors factorial arrangement, Randomized Complete Block (RCB) design with three replications. Factor A was 19 rice genotypes and factor B was two carbon sources (sucrose, maltose).

3.3.1.6 Data collection

1. Days to induce callus
2. Callus induction (%)
3. Callus induction increased by maltose
4. Callus induction (%) and callus induction increased by maltose were calculated by using the following formula-

$$\text{Callus induction (\%)} = \frac{\text{Calli produced (total no:)}}{\text{Anther plated (total no:)}} \times 100$$

$$\text{Callus induction increased by maltose} = \frac{\text{Callus induction \% (Sucrose)}}{\text{Callus induction \% (Maltose)}}$$

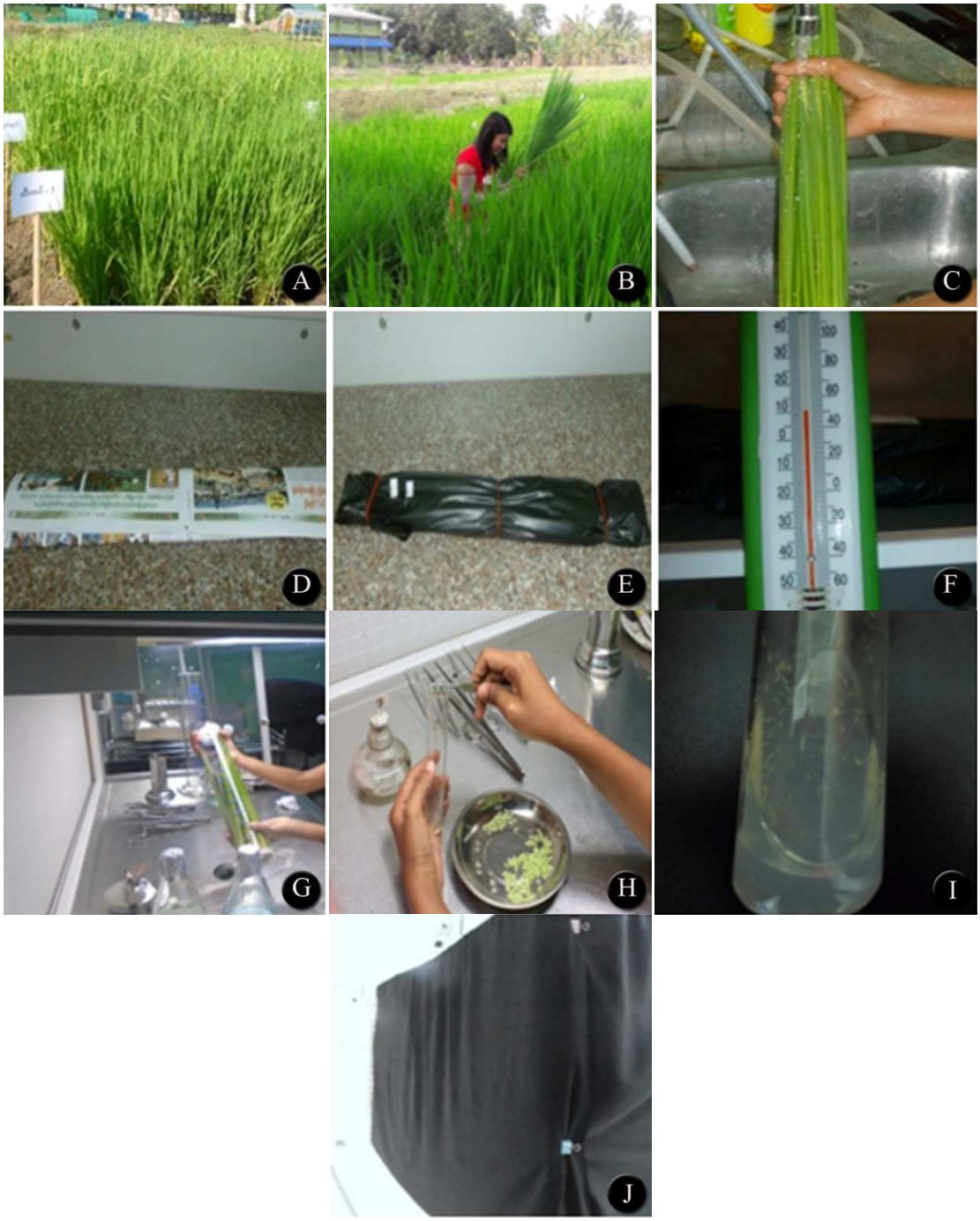

Plate 2 Protocol of anther culture : Callus induction

A. The 19 selected rice genotypes were grown in the field
B. Boots collection (Sources of explants)
C. Boots washing with tap water
D. Boots wrapped with papers
E. Boots covered with black plastics
F. Boots in refrigerators (10ºC for 8-10 days)
G. Sterilization of boots under aseptic condition
H. Anthers inoculation
I. Cultured anthers on N6 medium
J. Cultured test tube in dark condition for 13 weeks to induce callus

3.3.2 Plant regeneration

3.3.2.1 Preculture medium

Calli of each genotype (1-2mm) were transferred on “M” shaped paper bridge in culture test tube containing 5ml of N6 liquid medium with 2mg.L^{-1} 2,4-D and 0.5 mg.L^{-1} kinetin for two weeks.

3.3.2.2 Plant regeneration media

After two weeks on “M” shaped filter paper, the calli were transferred to MS solid media supplemented with 1 mg.L^{-1} kinetin, 1 mg.L^{-1}NAA and (0, 0.5, 1) mg. L^{-1} BAP, regeneration media.

In vitro shoots (5cm length) were transferred to basic MS medium for root regeneration. Well rooted plants were transferred to Yoshida’s nutrient solution for two weeks to allow root to develop vigorously. After two weeks, plants were transferred to small plastic cup for one week and then transplanted to pots and grown to maturity.

3.3.2.3 Culture condition

The test tube containing calli were incubated under continuous light at 25±1°C.Well rooted plants were acclimatized and grown in natural environment until maturity.

3.3.2.4 Experimental Layout

The experiment followed a two factor factorial arrangement, Randomized Complete Block (RCB) design with three replications. Factor A was 17 rice genotypes that induce callus and factor B was three different BAP concentrations (0, 0.5, 1) mg.L^{-1} with 1 mg.L^{-1} kinetin and 1 mg.L^{-1}NAA.

3.3.2.5 Data collection

1. Green spots formation (%)
2. Green plants regeneration (%)
3. Number of green plants per culture
4. Albino plants regeneration
5. Anther culturability

Green spot formation (%), green plant regeneration (%), albino plants regeneration (%) and anther culturability were calculated by using the following formula-

$$\text{Green spot formation (\%)} = \frac{\text{No.of callus produced green spot}}{\text{No.of callus plated}} \times 100$$

$$\text{Green plant regeneration (\%)} = \frac{\text{No.of callus produced green plants}}{\text{No.of callus plated}} \times 100$$

$$\text{Albino plant regeneration (\%)} = \frac{\text{No.of callus produced albino plants}}{\text{No.of callus plated}} \times 100$$

$$\text{Anther culturability} = \frac{\text{Total number of green plants}}{\text{No.of anthers plated test tube}} \times 100$$

3.4 Statistical Analysis

Analysis of variance was performed using Statix (version 8) software. Treatment means were compared by LSD at 5% level.

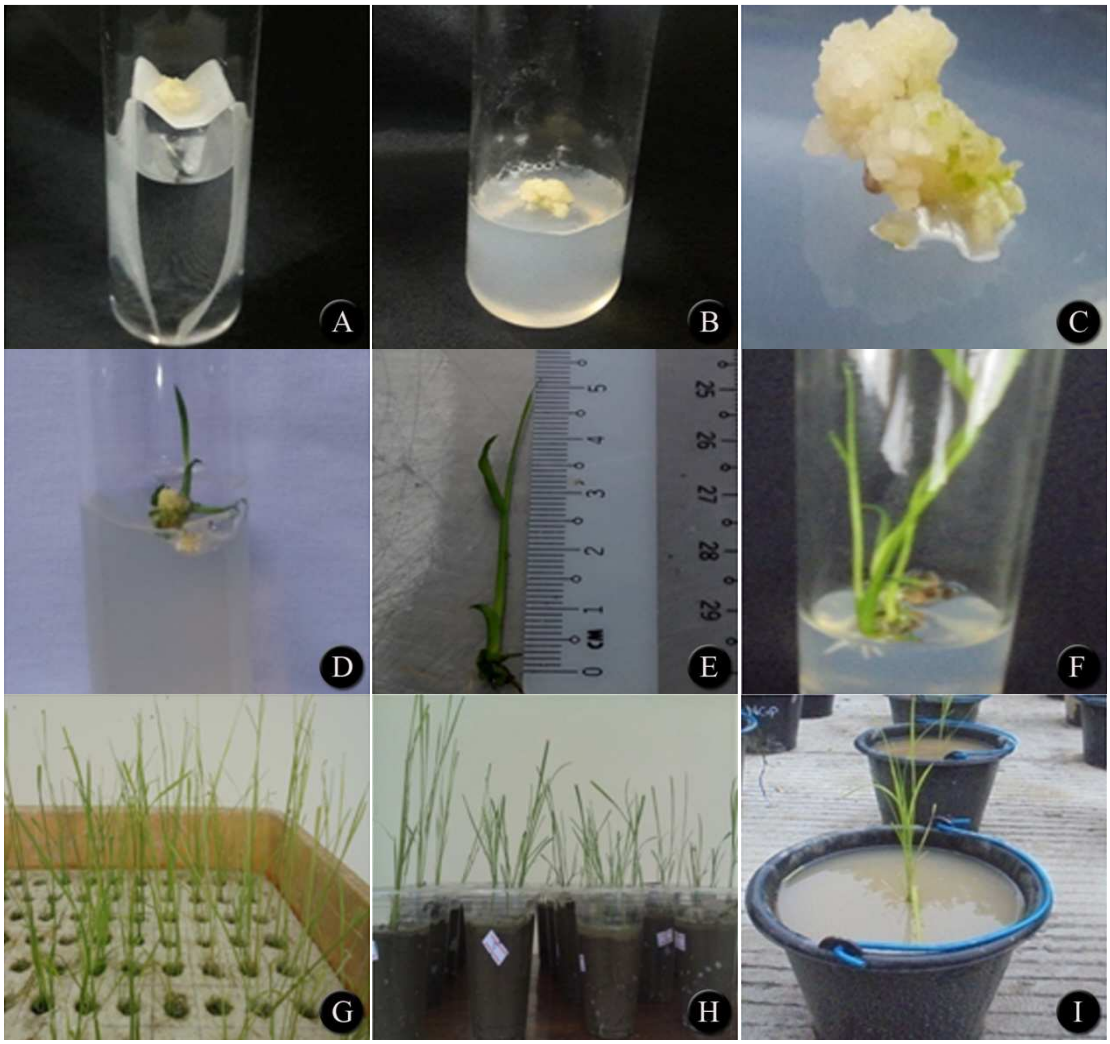

Plate 3 Protocol for anther culture: Plant regeneration

A. 21 days old callus on 'M' shaped filter paper of callus induction medium
B. 21 days old callus transferred on regeneration (MS) medium
C. In vitro green spots formation (5 weeks old callus)
D. In vitro green shoot formation
E. In vitro green shoot in 5 cm length
F. In vitro green shoot on basic MS medium for root regeneration
G. Green plantlets in Yoshida's nutrient solution
H. Green plant in plastic cup
I. Green plant in plastic pot

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Callus Induction

4.1.1 General description of callus induction

Most of inoculated anther changed color from yellow to light brown then dark brown within 1-2 weeks after cultured. It may be due to the transition of anther from gametophytic phase to sporophytic phase. This is similar with the results of Sengsai1 et al. (2007) who reported that anther browning is not surprising because many researchers also reported this changing of anther colors which is possibly due to the transition of gametophytic phase to sporophytic phase during androgenesis. In this experiment, it was observed that light brownish anthers produced callus. Yellow anther and dark brownish anther did not produced callus.

Plate 4 Callus induction in *in vitro* anther culture

A. Anthers on callus induction (N6) medium
B. Anther browning (14 days after culture)
C. Callus induction from anthers (28 days after culture)

4.1.2 Effect of genotypes and carbon sources on days to induce callus induction

The variation of days to induce callus formation as affected by two different carbon sources can be observed in table 4.1. It was found that there was not significant different between the two media (medium with sucrose and medium with maltose). Sin Thwe Lat produced callus at 26 days after inoculation on both media supplemented with sucrose and maltose. It is the earliest callus producing variety among the tested genotypes. Two genotypes; Shwe Pyi Htay and Thee Htat 3, did not produce no callus at all till 90 days after inoculation on both sucrose and maltose. However, four genotypes such as Thu Kha Hmwe, Khone Myint 2, Shwe Ta Soke and Hnan Kar produced callus on maltose at 27, 39, 43 and 59 days after inoculation respectively although these genotypes did not produce callus at all on sucrose till

90 days after inoculation. It may be due to different time requirement of genotypes for callus induction. Present results inline with the finding of Hearth and Bandara, 2011.Hearth and Bandara (2011) reported that the time requirement for the callus initiation was also genotype dependent and callus induction started 3 weeks after cultured.

According to their callusing response, tested rice genotypes can be classified into four different groups such as high (upper 5%), medium (from1.0% to 5%), low (lower 1%) and no response groups (Table 4.2). Genotypes of tropical japonica were included in high response on both media supplemented with sucrose and maltose. Thus, it can be said that tropical japonica has high response in callus induction of cultured anthers than indica. Silva and Ratnayake (2009) also reported that japonica types are known to be more responsive than indica types in anther culture. There were six genotypes; Shwe Pyi Htay, Thee Htat 3, Thu Kha Hmwe, Khone Myint 2, Hnan Kar and Shwe Ta Soke in no response group on media supplemented with sucrose. However, only two genotypes; Shwe Pyi Htay and Thee Htat 3 involved in no response group on media supplemented with maltose. Non sucrose responsive genotypes such as Thu Kha Hmwe, Hnan Kar and Shwe Ta Soke improved to medium group on media supplemented with maltose. Besides, one non sucrose responsive genotype; Khone Myint 2, moved to low group on maltose supplemented media. Moreover, maltose increased callus induction of Hmaw Bi 3 and Yebaw Sein from medium to high group. Thus, maltose can increase callusing response of most of tested indica genotypes. It may be due to positive effect of maltose on callusing response of rice genotypes. Khatun et al. (2012) reported that anther culture efficiency was enhanced in indica rice when sucrose was replaced by maltose.

Table 4.1 Days to induce callus formation of tested rice genotypes as affected by two different carbon sources

No.	Genotypes	Types	Days to induce callus	
			Sucrose	Maltose
1.	Yar-8	Indica	40	40
2.	Thee Htat Yin		39	40
3.	Shwe Pyi Htay		>90	>90
4.	Thee Htat -3		>90	>90
5.	Hmaw Bi 3		36	32
6.	Hmaw Bi 2		64	40
7.	Sin Thwe Lat		26	26
8.	Thu Kha Hmwe		>90	27
9.	Kone Myint 2		>90	39
10.	Shwe War Htun		34	34
11.	Lat Yone Kyi		52	48
12.	Hnan Kar		>90	59
13.	Yebaw Sein		44	44
14.	Shwe Ta Soke		>90	43
15.	Paw San Hmwe	Tropical japonica	48	45
16.	Paw San Taung Pyan Hmwe		40	40
17.	Paw San Bay Kyar		44	38
18.	Bay Kyar Taug Pyan		51	44
19.	Nga Kywe Taung Pyan		68	68

Table 4.2 Four different groups of rice genotypes classified according to their callusing response in anther culture

Carbon sources	High [1]	Medium [2]	Low [3]	No response
Sucrose	Paw San Bay Kyar	Hmaw Bi 2	Thee Htat Yin	Shwe Pyi Htay
	Paw San Hmwe	Yar 8	Shwe War Htun	Thee Htat 3
	Paw San Taung Pyan Hmwe	Hmaw Bi 3		Thu Kha Hmwe
	Nga Kywe Taung Pyan Hmwe	Sin Thwe Lat		Khone Myint 2
	Bay Kyar Taung Pyan	Lat Yone Kyi		Hnan Kar
		Yebaw Sein		Shwe Ta Soke
Maltose	Hmaw Bi 3	Thu Kha Hmwe	Thee Htat Yin	Shwe Pyi Htay
	Yebaw Sein	Shwe Ta Soke	Khone Myint 2	Thee Htat 3
	Nga Kywe Taung Pyan Hmwe	Shwe War Htun		
	Paw San Bay Kyar	Hmaw Bi 2		
	Bay Kyar Taung Pyan	Yar 8		
	Paw San Hmwe	Sin Thwe Lat		
	Paw San Taung Pyan Hmwe	Lat Yone Kyi		
		Hnan Kar		

[1] = (upper 5%); [2] = (from 1% to 5%); [3] = (lower 1%)

4.1.3 Effects of carbon sources on callus induction of tested rice genotypes

The effects of maltose on the callus induction of tested rice genotypes were described in table 4.3. Among the tested genotypes, Sin Thwe Lat and Bay Kyar Taung Pyan had better response in callus induction to sucrose while Thu Kha Hmwe, Khone Myint 2, Hnan Kar, and Shwe Ta Soke had specific response to maltose. It may be due to different requirement of carbon source to induce callus for tested rice genotypes. Swapna (2000) also reported that the effect of maltose and sucrose on androgenesis is variety specific. In the experiment, maltose increased anther response in most of tested genotypes (not only in indica but also in tropical japonica genotypes). It may be due to degradation of sucrose and maltose to glucose. This result agreed with Yi et al. (2003) who found that maltose increased anther response not only in indica but also in japonica. Sengsai et al. (2007) stated that maltose was found to be a better carbon source for callus induction in indica and japonica hybrid, BC1F1 (KDML 105//IRBB5/KDML105) anther culture compared with sucrose. Callus induction of Paw San Hmwe on maltose has 2.77 times than that of sucrose. Javed et al. (2007) also found that maltose had highest value for callus induction and the result of maltose was 1.5 times than that of sucrose. This is because slow rate of degradation of maltose to glucose as compared to sucrose. Slow degradation of maltose results in stabilization of medium osmolarity. Moreover fructose from the cleavage of sucrose inhibits the androgenesis (Last and Brettell 1990).

Table 4.3 Effects of maltose on callus induction in *in vitro* anther culture of different rice genotypes

No.	Genotypes	Types	Callus induction %		% increase in callus induction
			Sucrose	Maltose	
1.	Yar-8	Indica	2.11	2.33	1.10
2.	Thee Htat Yin		0.44	0.67	1.52
3.	Shwe Pyi Htay		0.00	0.00	0.00
4.	Thee Htat-3		0.00	0.00	0.00
5.	Hmaw Bi 3		2.44	6.56	2.69
6.	Hmaw Bi 2		1.11	2.22	2.00
7.	Sin Thwe Lat		2.78	2.67	0.96
8.	Thu Kha Hmwe		0.00	1.78	(~)
9.	Kone Myint 2		0.00	0.44	(~)
10.	Shwe War Htun		0.44	1.89	4.3
11.	Lat Yone Kyi		2.67	2.67	1.00
12.	Hnan Kar		0.00	2.44	(~)
13.	Yebaw Sein		2.56	6.78	2.65
14.	Shwe Ta Soke		0.00	1.78	(~)
15.	Paw San Hmwe	Tropical japonica	6.89	19.11	2.77
16.	Paw San Taung Pyan Hmwe		7.89	19.22	2.44
17.	Paw San Bay Kyar		6.67	11.78	1.77
18.	Bay Kyar Taug Pyan		10.00	7.44	0.74
19.	Nga Kywe Taung Pyan		7.00	7.22	1.03

4.2 Plant Regeneration

About 7 days old calli (1-2mm) were sub-cultured to “M” shaped filter paper in callus induction medium for two weeks and then moved to regeneration medium. After two weeks of sub-cultured, green spots were found on some calli and some produced albino plants while others turned to brown color. Callus browning may be due to non-embryogenic callus or media containing sucrose. Darachai et al. (2010) reported that media containing sucrose promote the ethylene production in plant tissue and ethylene is a plant growth regulator which can cause the browning of callus in tissue culture. The basic cause of albinism in rice is impairment of DNA in plastids or nuclei or in both of them (Kumari et al, 2009). Green spots formation and green plant regeneration are important for the success of *in vitro* anther culture. Most of differentiated calli produced either green or albino plants while some produced both.

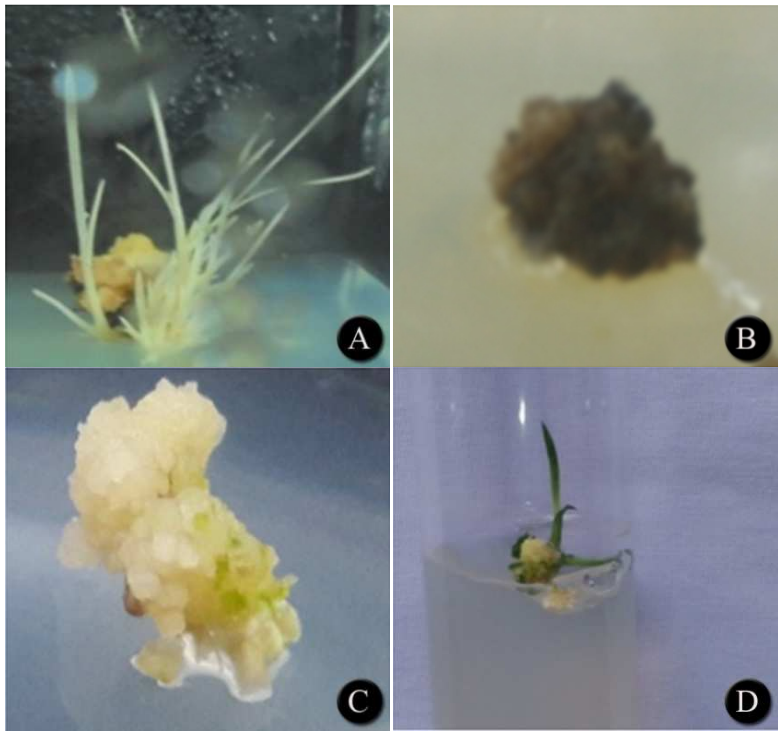

Plate 5 Shoot regeneration from callus of *in vitro* anther culture

A. Albino plant regeneration;
B. Callus browning;
C Green spot formation;
D. Green shoot regeneration;

4.2.1 Effects of genotypes and BAP concentrations on green spot formation

Effects of genotypes and different BAP concentrations on green spot formation were described in table 4.4. Only 10 genotypes gave callus producing green spots among 17 tested genotypes. Aung et al. 2015 also founded that only three of 12 tested japonica genotypes produced green plant although all genotypes induced calli. There was significant difference in green spot formation among genotypes. Thus, it was clear that green spot formation varied depending on genotypes. Highest green spot formation was found in Bay Kyar Taung Pyan (28.18%) among tropical japonica. However, It was not significant difference with Nga Kywe Taung Pyan (23.70%). Lat Yone Kyi (17%) gave highest green spot formation among indica rice. Green spot formation ranged from 1.48% to 28.15% among the tested rice genotypes. Kaushal et al. (2014) also proved that green spot regeneration varied with genotypes. Similar result was found in this experiment.

There was significant difference among BAP concentrations in green spot formation. Green spot formation varied with BAP concentrations. Highest concentration of BAP (1mg.L^{-1}) gave maximum percentage of callus producing green spot than the others. This might be because cytokinin causes development of single pole and formation of meristematic dome from which the shoot primordium develops (Sankepally and Singh 2016).

There was interaction effect between genotypes and different concentrations of BAP on green spot formation. Green spot formation of rice genotypes varied with concentration of BAP used. The effects of BAP concentrations on green spot formation of different rice genotypes were described in table 4.5. Green spot formation of Ye Baw Sein (4.45%) and Shwe Ta Soke (20.00%) were recorded only on media supplemented with 0.5 mg.L^{-1} BAP. However, most of tested genotypes produced maximum green spot formation on 1 mg.L^{-1} BAP.

Table 4.4 Effects of genotypes and BAP concentrations on green spot formation, green plant regeneration and number of green plants per culture

No.	Genotypes	Types	Green spot formation %	Green plant regeneration %	No. of green plants per culture
1.	Yar 8		6.67 c	2.22 fg	0.45 de
2.	Hmaw Bi 3		4.45 cd	0.00	0.00
3.	Hmaw Bi 2		5.93 cd	0.00	0.00
4.	Lat Yone Kyi	Indica	17.00 b	11.11 cd	3.22 a
5.	Hnan Kar		15.55 b	13.33 c	1.00 cde
6.	Yebaw Sein		1.48 d	0.74 g	0.11 e
7.	Shwe Ta Soke		6.67 c	5.93 ef	1.22 bcd
8.	Paw San Bay Kyar	Tropical Japonica	8.89 c	7.41 de	2.11 b
9.	Bay Kyar Taung Pyan		28.18 a	25.15 a	2.11 b
10.	Nga Kywe Taung Pyan		23.70 a	20.00 b	1.67 bc
	LSD (0.05)		4.55	4.75	0.64
	BAP concentrations ($mg.L^{-1}$)				
	0		7.11 c	5.11 b	1.36 ab
	0.5		12.89 b	10.00 a	1.96 a
	1		15.56 a	10.67 a	1.13 b
	LSD(0.05)		2.49	2.60	1.05
	Pr>F Genotypes		0.0001	0.0001	0.0001
	PGRs		0.0001	0.0001	0.0357
	Genotypes*PGR		0.0001	0.0005	0.0500
	CV %		40.71	52.55	74.45

Table 4.5 Effects of BAP concentrations on green spot formation of different rice genotypes

No.	Genotypes	Types	Green spot formation (%)		
			BAP concentrations ($mg.L^{-1}$)		
			0	0.5	1
1.	Yar 8	Indica	0.00	6.67	13.33
2.	Hmaw Bi 3		0.00	4.45	8.89
3.	Hmaw Bi 2		8.89	0.00	8.89
4.	Lat Yone Kyi		11.11	20.00	20.00
5.	Hnan Kar		13.33	13.33	20.00
6.	Yebaw Sein		0.00	4.45	0.00
7.	Shwe Ta Soke		0.00	20.00	0.00
8.	Paw San Bay Kyar	Tropical Japonica	6.67	4.45	15.56
9.	Bay Kyar Taung Pyan		13.33	35.55	35.55
10.	Nga Kywe Taung Pyan		17.78	20.00	33.33

4.2.2 Effects of genotypes and BAP concentrations on green plant regeneration

The effects of genotypes and BAP concentrations on green plant regeneration were presented in table 4.4. Only 8 genotypes produced green plants among the tested genotypes. There was significant difference among genotypes. One of tropical japonica genotypes in green plants regeneration, Bay Kyar Taung Pyan (25.15%) gave highest green plant regeneration among the tested genotypes and Hnan Kar (13.33%) produced maximum green plant regeneration in indica genotypes. Thus, it can be said that green plant regeneration was genotype dependent character. This was also reported by many researchers who studied on regeneration of cereal anther culture.

There was significant different among BAP concentrations in green plant regeneration (Table 4.4). In this experiment, green plant regeneration was highest in 1 $mg.L^{-1}$BAP (10.67%) and it was not significant difference with the result of0.5 $mg.L^{-1}$BAP (10.00%). Thus, addition of BAP to the medium with other hormones is more suitable for percentage of callus producing green plants than without BAP. Similar result was reported by Rout et al. (2016) that higher concentration of BAP along with low concentration of kinetin was found effective for shoot regeneration of anther culture.

There was significant interaction effect between genotypes and BAP concentrations on green plant regeneration (Table 4.4). Green plant regeneration of rice genotypes was different depending on concentration BAP used. Green plant regeneration varied from 0 to 25.15 % among the tested rice genotypes (Table 4.4). Ranjana et al. 1998 found that plant regeneration frequency varied from 0 to 26.86% depending upon the genotypes and the constituents of the media used. The effects of BAP concentrations on green plant regeneration of different rice genotypes were presented in table 4.6. Maximum green plant regeneration was found in 1 $mg.L^{-1}$ BAP for most of tested genotypes. However, green plant regeneration of Yebaw Sein (2.22%) and Shwe Ta Soke (17.78%) were recorded only on 0.5 $mg.L^{-1}$ BAP.

Table 4.6 Effects of BAP concentrations on green plant regeneration of different rice genotypes

No.	Genotypes	Types	Green plant regeneration (%)		
			BAP concentrations ($mg.L^{-1}$)		
			0	0.5	1
1.	Yar 8	Indica	0.00	2.22	4.44
2.	Lat Yone Kyi		6.67	13.33	13.33
3.	Hnan Kar		11.11	11.11	17.78
4.	Yebaw Sein		0.00	2.22	0.00
5.	Shwe Ta Soke		0.00	17.78	0.00
6.	Paw San Bay Kyar	Tropical Japonica	4.45	4.45	13.33
7.	Bay Kyar Taung Pyan		11.11	31.11	33.33
8.	Nga Kywe Taung Pyan		18.78	17.78	24.44

4.2.3 Effects of genotypes and BAP concentrations on number of green plants per culture

Effect of different genotypes and BAP concentrations on number of green plants per culture were presented in table 4.4. There was significant difference among genotypes in number of green plants per culture. Among the tested genotypes, Lat Yone Kyi gave maximum number of green plants per culture (3.22) and followed by Bay Kyar Taung Pyan (2.11) and Paw San Bay Kyar (2.11). Number of green plant in regeneration is genotype dependent character. There was significant different among BAP concentrations in number of green plants per culture. In the number of green plants per culture, (1.96) was highest in 0.5 $mg.L^{-1}$ BAP than other BAP concentration in the experiment. However, there was significant interaction effect between genotypes and BAP concentrations on number of green plants per culture. It means that the significant effect of genotypes also depend on the effect of BAP concentration.

The effects of BAP concentrations on number of green plants per culture of different rice genotypes were described in table 4.7. Higher number of green plant per culture for most of tested genotypes was found on media supplemented with 0.5 $mg.L^{-1}$ BAP. However, higher number of green plants per culture of Lat Yone Kyi (4.33) was recorded on media supplemented with 1 $mg.L^{-1}$ BAP than other BAP concentrations while Bay Kyar Taung Pyan (2.33) on media supplemented with 0 $mg.L^{-1}$. This clearly shown that there was the differential response of different rice genotypes to BAP concentrations. However, number of green plants per culture of Hnan Kar, Bay Kyar Taung Pyan and Nga Kywe Taung Pyan are non-responsive to BAP concentrations used in the experiment.

Table 4.7 Effects of BAP concentrations on number of green plants per culture of different rice genotypes

No.	Genotypes	Types	No. of green plants per culture		
			BAP concentrations ($mg.L^{-1}$)		
			0	0.5	1
1.	Yar 8	Indica	0.00	0.67	0.67
2.	Lat Yone Kyi		2.33	3.00	4.33
3.	Hnan Kar		1.00	1.00	1.00
4.	Yebaw Sein		0.00	0.33	0.00
5.	Shwe Ta Soke		0.00	3.67	0.00
6.	Paw San Bay Kyar	Tropical Japonica	1.67	3.33	1.33
7.	Bay Kyar Taung Pyan		2.33	2.00	2.00
8.	Nga Kywe Taung Pyan		1.67	1.67	1.67

4.2.4 Effects of different rice genotypes and BAP concentrations on albino plant regeneration

The effects of genotypes and BAP concentrations on albino plant regeneration were described in table 4.8. There was significantly different among genotypes on albino plant regeneration. Thu Kha Hmwe (26.67%) produced maximum albino plants among indica and Nga Kywe Taung Pyan (24.44%) gave highest albino plants among tropical japonica. Albino plant regeneration ranged from 8.89 to 26.67 % among the tested rice genotypes.

There was no statistically significant difference among BAP concentrations on albino plant regeneration. However, 0.5 $mg.L^{-1}$ BAP gave maximum albino plants formation (15.56%) in most of tested genotypes.

There was significantly different interaction between genotypes and BAP concentrations in albino plant regeneration. It means that albino plant regeneration of tested genotypes varied with BAP concentrations used.

The effects of BAP concentrations on callus producing albino plants of genotypes were presented in table 4.9. Albino plant regeneration of tested genotypes varied from 4.44% to 40% depending on BAP concentrations used. Talebi et al. (2007) stated that albino plant were morphological similar to the green plant except in chlorophyll deficiency and 5-100% albinos found in rice anther culture of their experiment.

Table 4.8 Effects of genotypes and BAP concentrations on albino plant regeneration

No.	Genotypes	Types	Albino plant regeneration %
1.	Thee Htat Yin		16.30 bc
2.	Hmaw Bi 3		8.89 d
3.	Hmaw Bi 2		12.59 cd
4.	Sin Thwe Lat		16.30 bc
5.	Thu Kha Hmwe	Indica	26.67 a
6.	Shwe War Htun		11.11 cd
7.	Latt Yone Kyi		9.63 cd
8.	Hnan Kar		10.37 cd
9.	Yebaw Sein		7.41 d
10.	Paw San Hmwe		20.74 ab
11.	Paw San Taung Pyan Hmwe		11.85 cd
12.	Paw San Bay Kyar	Tropical japonica	11.85 cd
13.	Bay Kyar Taung Pyan		9.63 cd
14.	Nga Kywe Taung Pyan		24.44 a
	LSD(0.05)		7.38
	BAP concentrations ($mg.L^{-1}$)		
	0		14.44
	0.5		15.56
	1		12.38
	LSD		3.41
	Pr>F Genotypes		0.0001
	PGR		0.1780
	Genotypes*PGR		0.0008
	CV %		105.3400

Table 4.9 Effects of BAP concentrations on albino plant regeneration of tested rice genotypes

No.	Genotypes	Types	Albino plant regeneration (%)		
			BAP concentrations ($mg.L^{-1}$)		
			0	0.5	1
1.	Thee Htat Yin	Indica	33.33	8.89	6.67
2.	Hmaw Bi 3		8.89	11.11	6.67
3.	Hmaw Bi 2		11.11	17.78	8.89
4.	Sin Thwe Lat		15.56	17.78	15.56
5.	Thu Kha Hmwe		20.00	40.00	20.00
6.	Shwe War Htun		16.33	15.56	4.44
7.	Lat Yone Kyi		8.89	15.56	4.44
8.	Hnan Kar		6.67	11.11	13.33
9.	Yebaw Sein		11.11	6.67	4.44
10.	Paw San Hmwe	Tropical Japonica	33.33	17.78	11.11
11.	Paw San Taung Pyan Hmwe		6.67	11.11	17.78
12.	Paw San Bay Kyar		8.89	13.33	13.33
13.	Bay Kyar Taung Pyan		4.45	11.11	13.33
14.	Nga Kywe Taung Pyan		20.00	20.00	33.33

4.2.4 Anther culturability of tested rice genotypes

The anther culturability of tested rice genotypes were shown in table 11. Bay Kyar Taung Pyan showed highest anther culturability (197%) among all tested rice genotypes. Among indica, Lat Yone Kyi had highest anther culturability (118%) although Hnan Kar gave maximum green plant regeneration (13.33 % in Table 4.4). This is because number of green plants per culture of Lat Yone Kyi (3.22 in Table 4.4) was higher than that of Hnan Kar (1.00 in Table 4.4) although green plant regeneration of Lat Yone Kyi (11.11% in Table 4.4) was lower than that of Hnan Kar (13.33% in Table 4.4). Bay Kyar Taung Pyan produced the highest number of green plants in this experiment. Anther culturability varied from 2 % to 197 % among the tested rice genotypes. Ouimio and Zapata also reported that anther culturability was found to be controlled by additive gene effects since 1990.Similar result was found in this experiment. Thus, anther culturability was considered as a genetically controlled character.

Anther derived fertile plants regenerated from eight different rice genotypes can be observed in appendix 4.

Table 4.10 Anther culturability of tested rice genotypes

No.	Genotypes	Types	Total no. of green plants grown in pot	No. of anthers plated test tube	Anther culturability
1	Yar 8	Indica	5	61	8
2	Lat Yone Kyi		47	40	118
3	Hnan Kar		8	34	24
4	Yebaw Sein		1	50	2
5	Shwe Ta Soke		9	51	18
6	Paw San Bay Kyar	Tropical Japonica	22	47	47
7	Bay Kyar Taung Pyan		55	28	197
8	Nga Kywe Taung Pyan		42	43	98

CHAPTER V
CONCLUSION

According to the results of this study, tropical japonica genotypes produced more callus than indica. Paw San Taung Pyan Hmwe was the highest (19.22%) among tropical japonica and Yebaw Sein (6.78%) was the highest among indica in callus induction. Maltose has superior effect to callus induction in most of the tested rice genotypes than sucrose. There was interaction effect between genotypes and carbon sources in callus induction. Therefore, it can be said that the effects of different carbon sources depend on different rice genotypes.

In regeneration, Bay Kyar Taung Pyan showed the highest (25.15%) among tropical japonica and Hnan Kar (13.33%) gave the highest among indica. In the case of BAP concentrations, combination of 0.5 $mg.L^{-1}$ BAP with 1 $mg.L^{-1}$ kinetin and 1 $mg.L^{-1}$ NAA, gave better results for most of the tested rice genotypes for green plant regeneration and number of green plants per culture, although combination of 1 $mg.L^{-1}$ BAP with 1 $mg.L^{-1}$ kinetin and 1 $mg.L^{-1}$ NAA was effective for green spot formation. An interaction effect was found between the effect of genotypes and BAP concentrations for green spot formation, green plant regeneration and number of green plants per culture. In anther culturability, the highest response was found in Bay Kyar Taung Pyan among tropical japonica and in Lat Yone Kyi among indica.

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APPENDICES

Appendix 1 Amount of different chemical components in stock solution for callus induction medium and plant regeneration medium

Components	Concentration in medium ($mg.L^{-1}$)	
	Callus Induction Medium (N6)	Plant Regeneration Medium (MS)
NH_4NO_3	-	1650
KNO_3	2830	190
$CaCl_2.2H_2O$	166	440
$MgSO_4.7H2O$	185	37 0
KH_2PO_4	400	170
$(NH_4)SO_4$	463	-
$FeSO_4.7H_2O$	27.85	27.88
Na_2EDTA	37.25	37.3
H_3BO_3	1.6	6.2
$MnSO_4.4H_2O$	4.4	22.3
$ZnSO_4.4H_2O$	1.5	8.6
KI	0.8	0.83
$Na_2MoO_4.2H_2O$	-	0.25
$CuSO_4.5H_2O$	-	0.025
$CoCl.6H_2O$	-	0.025
Nicotinic acid	0.5	0.5
Pyridoxin HCL	0.5	0.5
Thiamine HCL	1	0.1
Glycine	2	2.0
I -inositol	27.8	100

Appendix 2 Amount of chemical component s for stock solution of Yoshida's nutrient solution

Stock No.	Chemical	Amount (g or ml)/ 5 liters
1.	NH_4NO_3	457.000
2.	$NaH_2PO_4H_2O$	201.500
3.	K_2SO_4	357.000
4.	$CaCl_2$	443.000
5.	$MgSO_47H_2O$	1,620.000
6.	$MnCl_24H_2O$	7.500
	$(NH_4)_6Mo_7O_{24}4H_2O$	0.370
	H_3BO_3	4.670
	$ZnSO_4\,7H_2O$	0.175
	$CuSO_4\,5H_2O$	0.155
	$FeCl_3\,6H_2O$	38.500
	$C_6H_8O_7H_2O$	59.500
	1M H_2SO_4 (ml)	250

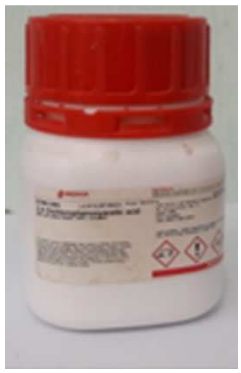
Appendix 3 Carbon sources, Agar, plant growth regulators used in the experiment

Maltose

Sucrose

Agar

Kinetin



2,4-Dichlorophenoxyacetic acid

1-Naphthyl Acetic Acid

Benzylaminopurine

Appendix 4 Fertile plants derived from *in vitro* anther culture of eight rice genotypes

Yar 8

Lat Yone Kyi

Hnan Kar

Yebaw Sein

Shwe Ta Soke

Paw San Bay Kyar

Bay Kyar Taung Pyan

Nga Kywe Taung Pyan