Establishment Of Efficient Anther Culture Techniques (For Haploid Breeding) Suitable To Indonesian Rice

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ABSTRACT

One of the objects of research that can be developed is anther culture. Plant regeneration derived from anther culture play an important role in supporting breeding program, especially hybrid rice breeding. Technically anther culture has been done in Indonesia however, the success of obtaining a population of double haploid lines desired still needs to be improved. The objectives of this research are to study the callus induction and plant regeneration, and to evaluate the efficiency of plant regeneration. The specific objectives of the program are: To optimize factors affecting anther culture, formulation media composition suitable and to anther culture of Indonesian rice cultivar, to establish general protocols of haploid breeding via anther culture in rice.

In preliminary experiment, genotypes of donor plant, developmental stage of microspore, duration of cold pre-treatment and physiological conditions during culture will be evaluate. Screening of varietal reaction to anther culture, evaluation will be conduct on callus induction and plant regeneration efficiency of 30 rice germplasms with different ecotype such as *japonica*, *indica* and *javanica/tropical japonica* germplasms.

The anther do not yet grow forming/induction callus. Therefore the anther cultured still in fresh condition after 20 days on medium. Little bit of anther looklike browning- color is brown. Plate examination after 20 days shows that no callus induction and number of anther browning was increased. Therefore some planting showed contamination by micro organism.

It has been cultured anthers of as many as 30 types/cultivars of rice. Cultivars have been planted include *indica* and *japonica* rice varieties, however, the most widely *japonica* group. There is incubation of anthere cultured, and up to now there is some callus growth and to regenerate form planlet. It has been cultured anthers of several types / cultivars of rice, most of the anthers do not grow to form callus. And some of callus are regenerated to form plants. However some anther culture is still in the incubation stage in a dark room with a temperature of 25^{o}C to form callus.

Key words: Anther culture, rice, rice panicle, mediumN6, cold pretreatment

Penentuan Efisiensi Teknik Kultur Antera (Untuk Haploid Breeding) Yang Sesuai Untuk Padi Indonesia

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Salah satu objek penelitian yang dapat dikembangkan adalah kultur antera. Regenerasi tanaman berasal dari kultur antera memainkan peran penting dalam mendukung program pemuliaan tanaman padi, terutama tanaman padi hibrida. Secara teknis kultur antera telah dilakukan di Indonesia, namun keberhasilan memperoleh populasi galur haploid ganda yang diinginkan masih perlu ditingkatkan. Tujuan dari penelitian ini adalah untuk mempelajari induksi kalus dan regenerasi tanaman, dan untuk mengevaluasi efisiensi regenerasi tanaman. Tujuan spesifik dari program adalah: untuk mengoptimalkan faktor-faktor yang mempengaruhi kultur antera, penyusunan komposisi media cocok dan kultur antera untuk padi Indonesia, membangun protokol umum pemuliaan haploid melalui budaya antera padi.

Tahun pertama, genotipe donor tanaman, tahap perkembangan microspore, durasi pre-Treatment dan kondisi fisiologis yang dingin selama kultur antera akan diamati. Skrining varietas berdasarkan hasil kultur antera dan evaluasinya akan dilakukan terhadap induksi kalus dan efisiensi regenerasi tanaman 30 germplasms padi dengan berbagai ecotype seperti japonica, indica dan japonica javanica tropis germplasms. Antera belum tumbuh membentuk induksi kalus. Oleh karena itu antera yang dikulturkan masih dalam kondisi segar setelah 20 hari pada medium. Beberapa antera mengalami *browning*-kecoklatan. Pengamtan setelah 20 hari menunjukkan bahwa belum ada induksi kalus dan jumlah antera kecoklatan meningkat. Oleh karena itu beberapa penanaman menunjukkan kontaminasi oleh organisme mikro.

Kultur antera telah dilakukan untuk 30 jenis/kultivar padi. Kultivar yang telah ditanami termasuk indica dan japonica varietas padi, namun, yang paling banyak adalah kelompok japonica. Hasil inkubasi kultur antera dalam ruangan yang gelap dengan suhu 25^{o}C menunjukkan induksi dan pertumbuhan kalus.

Kata kunci: Kultur antera, padi, malai padi, media N6, cold pretreatment

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EXECUTIVE SUMMARY

Introduction

One of the objects of research that can be developed is anther culture. Plant regeneration derived from anther culture play an important role in supporting breeding program, especially hybrid rice breeding. Technically anther culture has been done in Indonesia however, the success of obtaining a population of double haploid lines desired still needs to be improved. Application of this technique still needs improvement and development in both of genetically aspect such as preparation of plants as source of explants and technical potential which includes the modification of the media, equipments and skills. It can be done by referring to the information and research results which have been achieved.

Recently, anther culture technique has been widely used in breeding programs of many crops. The anther culture is one of the haploid breeding techniques that culture anthers in *in vitro* and obtain doubled haploid plantlets through androgenesis. A homozygous doubled haploid plant can be easily recovered by chromosome doubling of haploid plant. Doubled haploid offer many advantages to plant breeders with shortened breeding periods and its high efficiency in selecting useful recessive agronomic traits. The

most important factors of applying anther culture into practical breeding are ensuring appropriate number of regenerated plants from the F_1 or F_2 plants.

One of advantages of anther culture techniques that the protocols may permit the recovery of variants not easily obtained by conventional breeding practice. The probability for the in vitro recovery of benefit homozygous genotype reasonably high when anther culture, which provides the benefits of haploid event, is used to provide cells for biochemical selections. However, variation may be benefit or deleterious.

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.

The objectives of this research are to study the callus induction and plant regeneration, and to evaluate the efficiency of plant regeneration. The specific objectives of the program are: To optimize factors affecting anther culture, formulation media composition suitable and to anther culture of Indonesian rice cultivar, to establish general protocols of haploid breeding via anther culture in rice.

Methods

Research have been conducted at Plant Tissue Culture Department of Agronomy Faculty of Agriculture University of Jember. Periode of reserach conducting is from March till Mid of July 2014. Meanwhile the reserach already running at College of Agriculture and Life Science Kyungpook National University and it was started in 25 July 2014.

The research which have been conducted is part of first years program research with the following phases: the target is improvement skill of anther culture and medium composition suitable for anther culture.

In preliminary experiment, genotypes of donor plant, developmental stage of microspore, duration of cold pre-treatment and physiological conditions during culture will be evaluate. Screening of varietal reaction to anther culture, evaluation will be conduct on callus induction and plant regeneration efficiency of 30 rice germplasms with different ecotype such as *japonica*, *indica* and *javanica/tropical japonica* germplasms.

Experiment in which running at KNU Korea is set up in the *screen house* and rice field using 30 genotypes of *O. sativa*. Pre-germinated seeds from each genotype are sown

in pots. Collection of anther Plants are then replanting on the paddy field and watered. Nitrogen as urea, phosphorus and potassium are used to fertilize plants. Anthers are collected at the early flowering stage, when young panicles still enclosed within the leaf sheath. Selection is based on a maximum distance between the auricle and the next subtending leaf of 5-6 cm for *japonica* and 7-10 cm for *indica*. Expected this coincides with the mid-uninucleate stage which is most responsive to anther culture.

Panicles are collected from plants between 9:00 – 10.00 h in the morning and are washed with tap water. After clipping the flag leaves, panicles are sprayed with 70% ethyl alcohol. There are two kinds of Cold Pre-treatment: First method is ather is plated BEFORE Cold Pre treatment- after plated then the anther treated by Cold Treatment for 12oC and 15 days. Panicles collected then are sprayed with alcohol 70% and the spikelet's pull out and each spikelet are cutted at base part to free the anthers from the filaments. Using forceps pointy the individual spikelt "knocked" on the rim of Petri-dish. The anther then plated onto Petri dish containing medium N6 (Chu et al., 1975). Immature anthers are cultivated in Petri dishes containing 25 ml of Chu et al., (1975) N6 medium supplemented with 2 mg/l NAA and 2 mg/l; 0.2 mg/l kinetin and ABA 2 mg/L . Medium is solidified with 5% Gelrite.

Petri dishes containing anther and callus medium are then placed in dark room with temperature 12oC for 15 days. It is cold pretreatment directly on anther before incubated at 25±1ºC in the dark to develop organogenic callus. The N6 medium contained: Macro (mg/l): KNO3(2830,(NH4)2SO4 (463), MgSo4.7H2O (185), KH2PO4 (400), CaCl2.2H2O (166). Micro (mg/l); KI (0.8), H3BO3 (1.6), MnSO4 4H2O (4.4), ZnSO4.7H2O (1.5), Na2MoO4.2H2O (0.250), Na2EDTA.2H2O (37.25), FeSO4.7H2O (27.85) Organics: Nicotinic acid (0.5), Pyridoxin-Hcl (0.5), Thimine-HCl (1.0). Carbohydrate: Sucrose 20 (g/l) and Glukose (10 g/l).

Second method is the panicles collected are sprayed with alcohol 70% and kept in polyethylene bags with vacuum and incubated in the dark at 12ºC for 15 days, to optimize the cold treatment duration harvested spikes containing early to mid for15-days. Anther then will be platted on medium AFTER Cold Pretreatment. We have try to inoculated anther on medium for 30 type. We selected the young panicle which the panicles with boot leaf sheath. Selection was based on maximun dictance between the auricle and the next subtending leaf of 3-5 cm for *japonica* type and 4-6 cm for *indica* type.

Results and Discussion

Result: the anther do not yet grow forming/induction callus. Therefore the anther cultured still in fresh condition after 20 days on medium. Little bit of anther looklike browning- color is brown. Plate examination after 30 days shows that no callus induction and number of anther browning was increased.

We have cultured anther use diferent type/varieties, and use different hormon supplemented on medium. Therefore the result shows that there is no callus induction.

A B C

Figure 1. Cultured anther varieties of Inpari 4 date of July 1st on medium C (A); anthers enlarged image (B); Anther culture dated May 3rd varieties Inpari 7 on medium B (C)

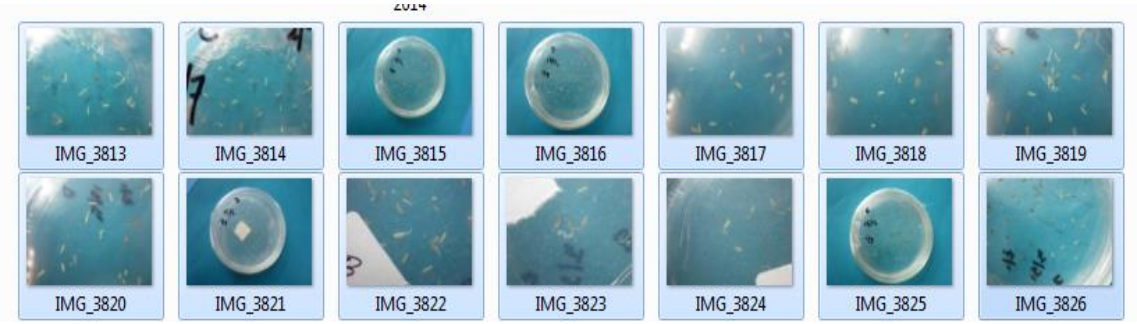


Figure 2. Cultured anther varieties of IR 64 on medium N6 supplemented by some plant hormone

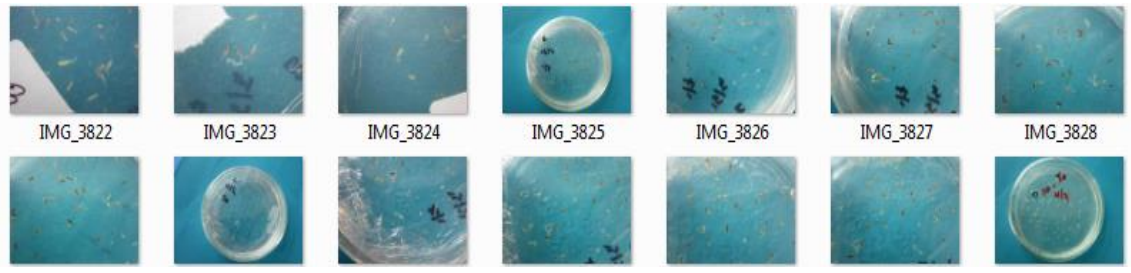


Figure 3. Cultured anther varieties of Inpari Sidenuk on medium N6 supplemented by some plant hormone

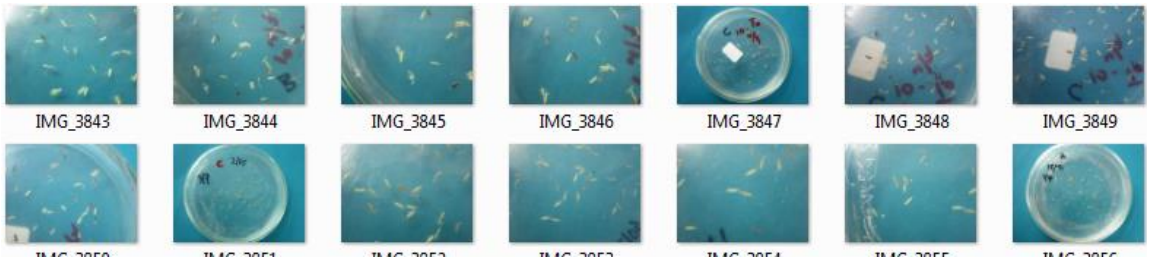


Figure 4. Cultured anther varieties of IR 10 on medium N6 supplemented by some plant hormone

Observation using a microscope shows that cultured anthers seem a bit "ballooned" and anther cells appear to grow slightly. Anthers cultured tend to be very slow to grow and form a callus (Figure 2). The problem of contamination of anther culture is not much. Some combination of several plant hormones already used, however observations show the same results.

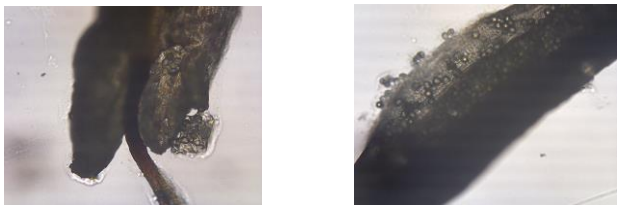
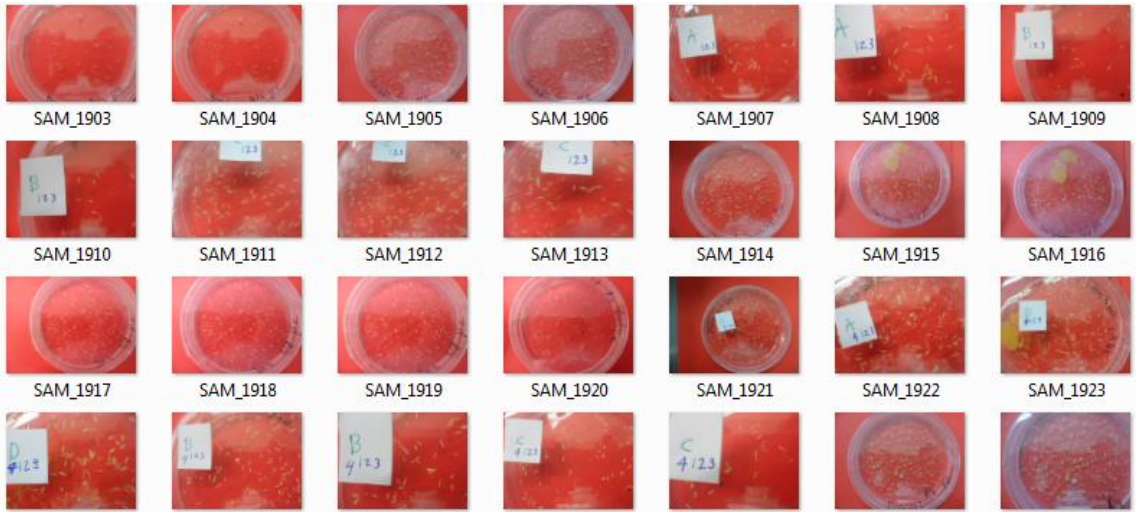


Figure 5. Microscopic observation on anther culture.



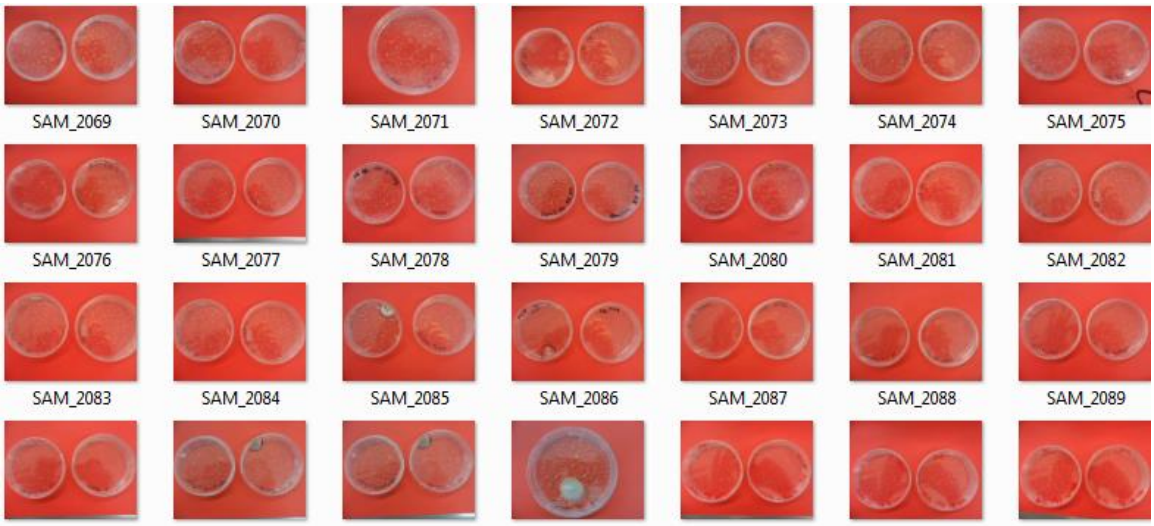
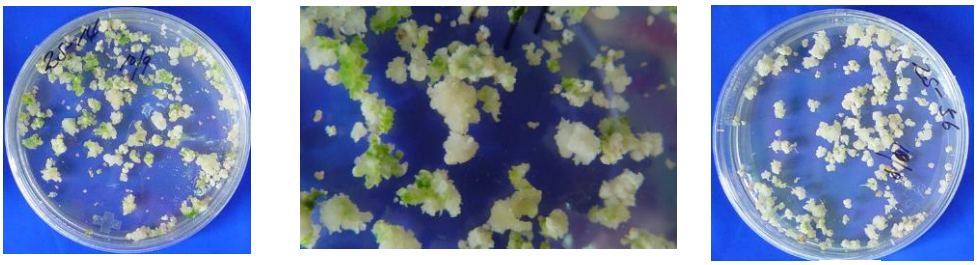


Figure 6. Cultured anther of some rice type on medium N6 supplemented by some plant hormone



A B C

Figure 7. A) Callus of anther of rice milyang on MS supplemented by some plant hormone
B) Callus of anther of rice Samgang on medium MS supplemented by some plant hormone
C) Callus of anther of rice Seolgaeng on medium N6 supplemented by some plant hormone

Figure 8. A close view of plant regeneration from callus of type rice of Hopum and Samgang on medium of N6 + 2 mg/l Kinetin + 0.2 mg/l IAA

Callus began to form after anther culture incubated 6 weeks. The process of initiation of callus formation occurs within the anther. Suggested that metabolites absorb in anther space and provide nutrients for the development of microspores and surrounds the embryo or young callus formed

Among 30 types of different rice anther some grow into callus. But most cannot grow into anther callus because a lot of contamination. Suspected contamination comes from grains of rice that contains a lot of water. Water is believed to contain many micro-organisms. Callus obtained produce or cannot regenerate plants. Growing callus was transferred to N6 medium with the addition of 2 mg / l Kinetin + 0.2 mg / l IAA.

According to Chung (1992) on rice anther culture generally potentially embryo genetic callus formed at the age of three to eight weeks after inoculation anther. Callus produce green plants, which in turn will be grown-regenerated as single plant on MS medium. Callus which first appeared generally easier to regenerate plants.

To define the most effective carbohydrate source in rice anther culture, carbohydrate used showed to be the preferential carbon source for the callus induction. The ratio of callus induction was from 2.5% to 3.5 %. Jain et al. (1996) report that carbohydrate was preferential carbon source on the shoot regeneration in rice protoplast culture. The most common source of carbon source, sucrose, is decomposed into glucose and fructose by enzymatic hydrolysis of invertase secreted by rice cells. Since carbohydrate is compose of two molecules of glucose, the high ratio of calli induction in carbohydratemedium might be due to diauxic growth pattern of rice cell. The callus induction ratio was enough high in the carbohydrate containing medium supplemented one among the rice ecotype. The indica rice inpari 4, 1.5% of anthers produced callus in medium containing carbohydratebut only 0.2% of anthers produced callus in the medium supplemented with sucrose plus glucose. Interestingly, the increasing ratio of callus induction was higher in *Tongil* and *Indica* than in *Japonica* varieties. Carbohydratewas effective in the plant regeneration as a carbon source, Jain et al (1996) reported similar results in plant regeneration of protoplast-derived callus. From these results, we suppose 2 possible hypotheses: 1) carbohydrate activates a certain gene which enhances anther culture responses in *indica* and *japonica* or 2) an unknown gene in *indica* rice using carbohydrateas a carbohydrate source may act different when it uses sucrose.

The process of cold shock is one of the most important factors of promoting callus induction and plant regeneration in anther culture. Vacuum packaging of immature panicles during cold shock period was compared to that of conventional open-air storage. The callus induction was increase to 24 to 34 percent higher than in open-air storage. In conventional storage, after 30 days the callus induction was lowered to 11.8 percent than in 15.3 percent of 15 days. On the other hand, callus induction was increased from 9.0 percent to 15.3 percent with prolonged store period to 30 days in vacuum packaging. The promotion of callus induction after 30days storage in vacuum packaging might be concerned to deteriorating of panicles and anthers. After 30days of storage, many of panicles turned into black to gray color but in vacuum packaging, most of panicles kept green color. For the practical use of anther culture into breeding, it is very difficult to keep the exact duration of cold pretreatment due to the concentration of the heading date of elite line. These results may give benefits of concentration of inoculating anthers. The combining effects of carbohydrateand vacuuming packaging on the callus induction and plant regeneration in different ecotypes of rice anther culture.

It has been cultured anthers of several types / cultivars of rice, most of the anthers do not grow to form callus. And some of callus are regenerated to form plants. However some anther culture is still in the incubation stage in a dark room with a temperature of 25^{o}C to form callus.

As suggestions it should be note that to obtain callus that will be used for new plants regeration, it needs to be cultured anthers of several types of rice plants. In culture antheres it should be done using the anther culture medium with addition of carbon source. For optimization callus induction from anther it should be selected and use young anthers. Use anthers from the middle of the panicles.

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