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Somatic embryogenesis of East Kalimantan local upland rice varieties

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Abstract. Somatic embryogenesis is the formation, growth and development of embryos from somatic cells. Somatic embryo induction is one of the in vitro plant propagation techniques that is very important for plant developmental purposes. Four local upland rice varieties of East Kalimantan, Mayas Pancing, Gedagai, Siam and Serai, were used in this study. A total of 200 explants (mature rice grains) for each varieties were inoculated on MS solid medium supplemented with 1 mg L⁻¹ 2,4 Dichlorophenoxy acetic acid (2,4-D) and 0.5 mg L⁻¹ 6-Benzylaminopurine (BAP). The results showed that response of each variety differed to embryomatic induction, indicated by callus induction rate and callus quality, in terms of callus color and structure. The fastest callus formation was soberved in Gedagai variety (8 days) while Mayas Pancing (13 days) was the latest one. The rate of callus induction varied from 60 to 98.5 %, and Serai variety has the highest callus induction rate. The highest friable callus structure was found in Siam variety (89.1%) and the lowest was in Gedagai (62.5%). Callus color was dominated by the yellowish-white (transparent) on all varieties tested. Most of the callus was potential as embryogenic callus characterized from the nodular and globular of friable callus structure and its yellowish-white color.

1. Introduction

Research on in-vitro culture has been rapidly developed in higher-plant cells and tissues. Widespread application of tissue culture technologies to crop improvement have been reported included in cereal species such as rice. Organogenesis and embryogenesis are types of cells differentiation in the regeneration of in vitro derived plants. Organogenesis is characterized by the unipolar bud primordial with subsequent development of shoot and roots. On the other hand, the bipolar structure, having both shoot and root apical meristem in a single cell, is the character of embryogenesis pathway [1-2].

Somatic embryogenesis is a somatic cell that develop to form new plant through specific embryonic developmental stages without gamete fusion [1, 3]. Embryogenesis begins from a single cell then proliferates with differentiating organs and tissues. Embryogenesis is the most popular plant regeneration due to its prospect for specific plant developmental purpose, such as genetic transformation, cryopreservation, synthetic or artificial seeds, protoplast production for somatic hybridization, and somaclonal variation.

Various factors critically influence somatic embryogenesis, including plant genotype, explant source, media composition, and plant growth regulator [4]. The use of growth regulating hormones



should consider the type and concentration of the substances in accordance with the plant genotype as well as physiological conditions of explant, since each genotype and plant tissue has specific response to the provision of growth regulators. The successful induction of somatic embryos and subsequent recovery of viable plants is not routine or efficient for the majority of species. Highly genotypic variation resulting in specific morphogenetic response is a major limitation in rice embryogenesis. Therefore, optimization of embryogenesis protocol for desired genotype is essential.

Somatic embryogenesis using East Kalimantan local upland rice variety has not been reported. The local rice variety carry several important traits based on previous studies [5-6], and potential to be developed as new superior rice variety in plant breeding program including plant biotechnology approach. Therefore, the application of somatic embryogenesis in East Kalimantan local upland rice variety can be utilized for genetic quality improvement through genetic manipulation, genetic engineering and increasing genetic diversity of rice through somaclonal variation.

2. Materials and methods

2.1. Plant materials

Four local upland rice varieties from East Kalimantan, Serai Gunung, Gedagai, Mayas Pancing, and Siam were used in this study. These varieties were included into superior local rice varieties having good grain quality in terms of taste characteristics. The mother plants were grown in the screen house and maintained according to general upland rice cultivation procedure.

2.2. Explants and Media Preparation

Mature embryos were used as explants source in this study. The embryos is derived from a physiologically mature rice grain obtained from the ready harvested plants. The grains were selected with the following criteria, free from pests and diseases, and the grains size were uniform.

A total of 200 explants were used for each variety. The selected rice seeds were cleansed using detergent and rinsed with distilled water. The lemma and palea of the seeds were removed manually. The caryopsis then were surface sterilized in 70% alcohol solution for 1-2 minutes, then with 30% of sodium hypochlorite for 15 minutes and followed with 10% of sodium hypochlorite for 5 minutes. Explants finally were washed with sterile distilled water twice for 3-5 minutes and drained on sterile filter paper. The sterilization steps were conducted in LAFC (Laminar Air Flow Cabinet).

2.3. Inoculation and Somatic Embryos Induction

Somatic embryos were induced by inoculating the sterilized seeds explant on the callus induction medium. The callus induction medium was agar solidified MS [7] media containing sucrose (30 gL⁻¹) and supplemented with 1 mgL⁻¹ 2,4-D and 0.5 mgL⁻¹ BAP. Inoculation was conducted by lying the explants horizontally on the media. The cultures then were incubated in dark condition (25±2 °C) for somatic embryos induction.

3. Results and discussion

3.1. Callus Formation

The rate of callus formation varied in different plant genotypes (Figure 1). The fastest callus formation was observed in Gedagai variety which needs only 8 days for callus formation, and Mayas Pancing was the latest one. Variation was also observed in the callogenesis response among the four varieties (Tabel 1). The percentage of explants forming callus varied from 60-98.5%. Among the genotypes, Serai variety produced the highest explants forming callus. On the other hand, Gedagai which was the fastest in forming callus has the lowest ability of explants forming callus.

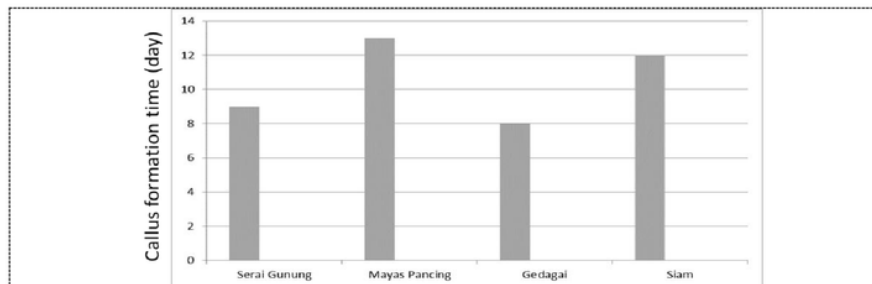


Figure 1. Callus formation time of four local upland rice varieties of East Kalimantan.

Table 1. Number of explants forming callus in four local upland rice varieties of East Kalimantan^a.

Variety	Explants forming callus (Mean \pm SD ^b)	Percentage (%)
Serai	197 \pm 0.31	98.5
Gedagai	120 \pm 1.09	60.0
Mayas Pancing	138 \pm 0.98	69.0
Siam	169 \pm 0.88	84.5

^a Data was observed from 200 explants.

^b SD=Standard Deviation.

Each variety has different ability of callus induction in somatic embryogenesis as observed in this study. This indicates that the ability of embryogenesis is influenced by the genotype of the plant. Significant genotypic differences in callus induction using mature embryo explant in rice somatic embryogenesis have been reported earlier [8-9]. Genetic variation in each genotypes is resulted by the different levels of endogenous phytohormones influencing callus induction and proliferation [10]. Therefore, somatic embryogenesis is not only a routine techniques using standard use of media to promote the induction and development stages of embryo, since the successful induction of somatic embryos is not always efficient for all plant species even within species.

The initiation of callus in mature embryos with endosperm-supported explant developed from scutellar tissue layer, the tissue between endosperm and zygotic embryo (Fig. 2A). Then the callus continued to grow as a result of the proliferation of callusing cells (Fig. 2B), forming a larger mass of cells. Calli induced from scutellar tissue of mature seed are the excellent source of cells for in vitro regeneration and for the production of transgenic rice [11-12]. Therefore, when using endosperm without zygotic embryo as an explant source in rice somatic embryogenesis, the removing process of zygotic embryo should be as thoroughly as possible that it does not take away the scutellar layer together.

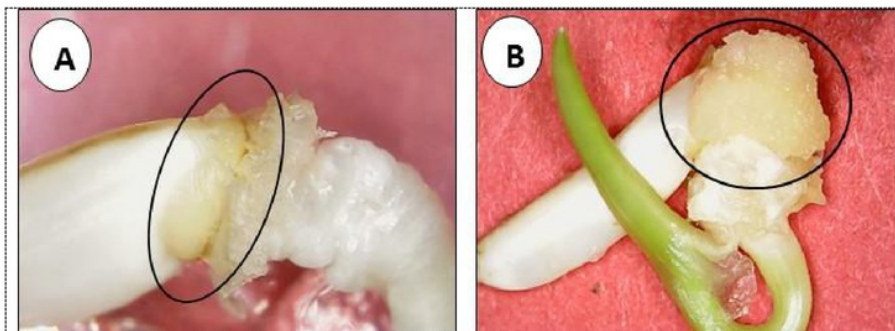


Figure 2. Initial stage of callus development in mature embryo with endosperm supported explant; A. Early formation of callus in the scutellum tissue; B. Callus enlarged forming a larger mass of the cells.

3.2. Callus Structure

Callus structure observation was carried out to evaluate whether the cell structure was compact or not. The compact callus structure is the composite callus consisting of closely packed cells. The non-compact callus structure is the crumbling one or friable (do not converge between the callus composite cells) (Figure 3). The callus structure observation of the four local upland rice varieties of East Kalimantan is presented in Table 2. The callus structure was dominated by the non-compact callus structure. The highest non-compact callus structure was found in Serai variety in which around eighty percent of the calli are friable, while the highest compact callus structure was observed in Gedagai variety.

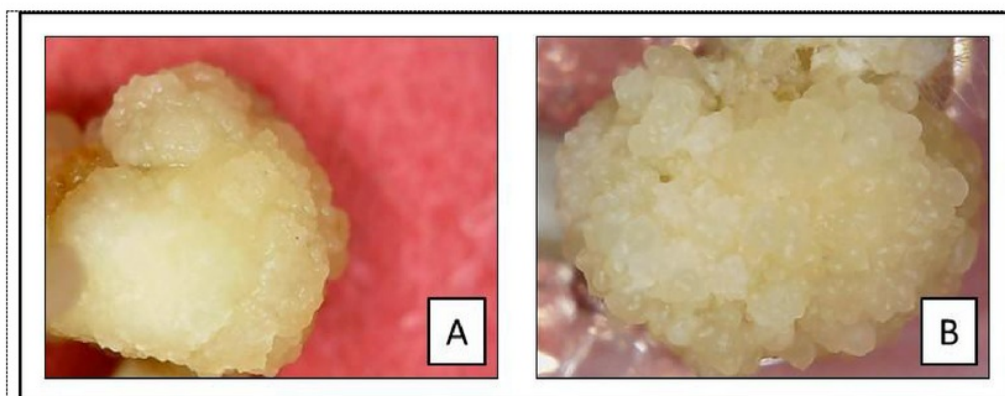


Figure 3. Differences of non-compact and compact callus structure. A. Compact callus structure; B. non-compact callus structure.

The quality of a callus can be observed from the callus structure. The good quality callus is assumed to have a non-compact structure (friable). The friable callus structure is embryogenic potential. The embryoid development was abundant in this structure and plants were easily regenerated [13]. There was a striking difference between friable and compact callus from the

microscopical sections. Vascular bundles which were prominent in compact callus, were not present in friable callus [14].

Table 2. Callus structure of East Kalimantan local upland rice variety.

Variety	Compact (Mean \pm SD)	Percentage (%)	Non-compact (Mean \pm SD)	Percentage (%)
Serai	34 \pm 0.86	17.3	163 \pm 0.89	82.7
Gedagai	45 \pm 0.69	37.5	75 \pm 1.04	62.5
Mayas Pancing	40 \pm 0,61	29.0	98 \pm 1.05	71.0
Siam	17 \pm 0.48	10.1	152 \pm .75	89.1

It was observed that the friable callus was initially a compact form of invisible nodules of the embryo (Figure 2), after two weeks the compact callus began to form nodules of the embryonic candidate (3B), depending on the variety and combination and concentrations of growth regulator substances. The next stage, is the nodules formation having a sphere of smooth and thin walls on the surface of the cell which is called as globular phase (Figure 3B). The smooth and glossy surface of the callus is when the somatic embryo of the globular phase begin, whereas in the compact callus structure, the surface of the callus does not form nodules (Figure 3A).

3.3. Callus Color

Callus growth indicator on in vitro culture can be observed from callus color. Callus color depicts the visual appearance of the callus, it indicates whether a callus is healthy and good growing or degenerate. Visual observation of the callus color of the local upland rice somatic embryos is presented in Table 3. There were yellowish-white or creamish, white and brown callus color. The callus color is dominated by the yellowish-white color (transparent), ranging from 77.5 to 87.6 % depending on the plant genotype. The highest number of yellowish colored callus was found in Serai variety, while Gedagai variety produced the lowest one.

Besides callus structure, callus color also describes the regeneration ability of the cells in forming buds and roots [4]. The yellowish-white color is one of the embryogenic callus characteristic. Nodular and globular callus with transparent color (pale yellow) (Figure 3B), usually have a higher ability to form buds than the compact and browning colored callus [15].

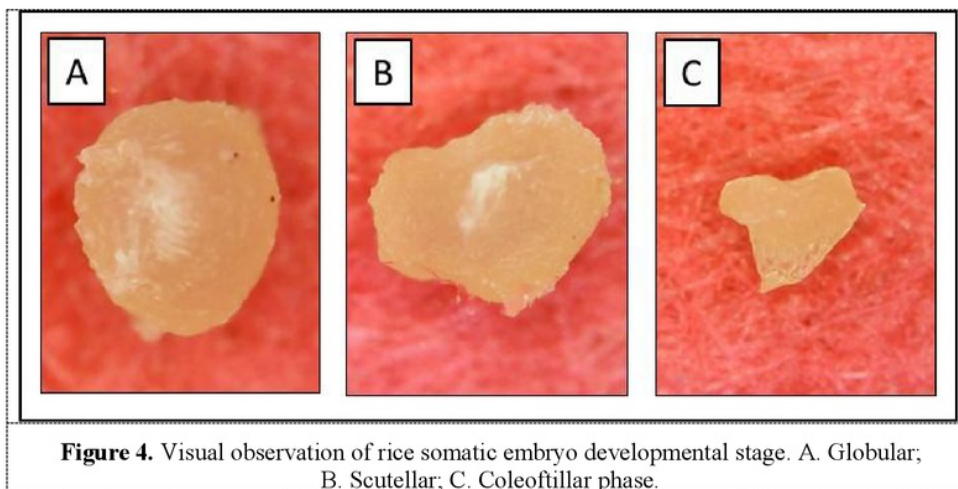
Table 3. Callus color of East Kalimantan local upland rice varieties.

Variety	Yellowish-white (Mean \pm SD)	White (Mean \pm SD)	Brown (Mean \pm SD)
Serai	165 \pm 0,83	13 \pm 1,01	19 \pm 0,46
Gedagai	93 \pm 1,04	13 \pm 0,95	15 \pm 0,38
Mayas Pancing	112 \pm 0,94	14 \pm 0,28	10 \pm 0,0
Siam	148 \pm 0,77	10 \pm 0,0	13 \pm 0,29

It was observed that the brown callus color present in low percentage in this study. The brown color of the callus indicates the degeneration or cells growth declining, and even may lead to death [16]. It is a type of non-embryogenic callus [13], and has low ability of regeneration. Brown callus occurs frequently in compact callus structure, indicated by lignification of the browning cells [17]. The brown callus was initially white, then the color changes to brown.

3.4. Embryosomatic Developmental Stage

Somatic embryos are bipolar propagules, derived from somatic tissue but the development, morphology and physiology are similar to sexually derived zygotic embryos [1]. The development of embryo occurs through several phases. There is a slight difference between the somatic embryo developmental stage of dicot and monocot plants. In dicotyledonous plants, the stages are globular, heart, and torpedo [18], whereas in monocots, the stages are globular, scutellar, and coleoptillar [15, 19]. In this study we captured the somatic embryos developmental stages of East Kalimantan local upland rice variety (Figure 4) representing the globular, scutellar, and coleoptillar stages. Based on morphological observations on the callus, the most frequently callus observed is the globular phase (Figure 4A). The scutellar and coleoptillar developmental phase was rarely found (Figures 4B and 4C).



The success of somatic embryogenesis occurs when the callus or cell is embryogenic, characterized by bipolar structures, having both basal and apical meristem initiating root and shoot development simultaneously [2]. In monocots, apical and basal cell lineages are usually incorporated into a pear-shaped proembryo and are difficult to distinguish from each other [20], which might be described as the scutellar and coleoptillar developmental of stage (Figure 4B and 4C).

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