Effect of Medium Compositions on The Growth of Rice (Oryza sativa L. cv. Ciherang) Callus

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Abstract— The efficiency of in-vitro regeneration of rice is spesific, which means a suitable medium for the regeneration of one variety may not be similar to other varieties. In this study, eksplan was derived from sterile radical of Ciherang var. This experiment consist two steps. First, induction of callus, and the second step was callus regeneration. The formulation for calli induction consists of three levels, such as i1 (2.4 D 2 ppm), i2 (2.4 D 3 ppm + BAP 0.25 ppm + Casein Hydrolisate 300 ppm + Proline 600 ppm) and i3 (2.4 D 2 ppm + Kinetin 0.5 ppm + Casein Hydrolisate 500 ppm + Proline 500 ppm) with N6 as a basic medium. Callus generated from those media then used in the next experiments for shoot regeneration which consists of two factors, the first factor was concentration of hormones BAP (r) (1 ppm, 2 ppm) and the second factor was concentration of the hormone Kinetin (k) (1 ppm, 2 ppm) with MS as a basic medium. The results showed that the highest embryogenic callus was obtained from i3 treatment by 62,96%. The fastest rate of greeny callus and the emergence of shoots were found in i2r2k2 and i2r2k1 treatments. The results indicated that the media supplemented with high concentration of Kinetin (2 ppm) was more effective to induce the greeny of callus, and slightly lower concentration (1 ppm) leads to the formation of shoots.

Key Words—Ciherang, embriogenic calli, in-vitro, shoot regeneration, kinetin.

INTRODUCTION

Rice (*Oryza sativa* L.) is the world's single most important staple food crop, and it represents a primary food for Indonesian society, which is difficult to replace with other foodstuffs from other commodities. Along by increasing the population, agricultural land becomes narrow and converted into residential and industrial area, therefore fulfillment of the needs to be greatly reduced. In addition, the effects of natural disasters and pest attacks caused the rice production dramatically decreased [1].

Many researches have been done in an effort to improve the quality and quantity of rice seed, one of which is the application of techniques of in vitro culture. In vitro culture is a method of plant propagation using the explants were planted in a particular medium that can produce callus in large quantities [2]. In vitro culture can also be used as a method for rejuvenation of rice seeds which expected to be free from diseases. Application of in vitro propagation of rice for traits improvement are considered to get high-yielding of rice, resistant to pests and diseases through biotechnology of gene transformation.

Previous studies were done on rice subspecies japonica and javanica in induction of callus through the in vitro culture. However, little information obtains concerning to the propagation of rice subspecies indica [3]. The regeneration using in vitro techniques in subspecies indica is still low and the process can not be repeated (*reproducible*). Regeneration of rice is specific, which means a suitable medium for the regenerate of one variety may not be similar to other rice varieties.

In this study, we tried to find the optimum medium for the embriogenic callus induction and regenerated of callus cv.Ciherang.

MATERIALS AND METHODS

This experiment consists two steps: (1) Callus induction, (2) Shoot regeneration .

a. Callus Induction

The seed explant was prepared from cv. Ciherang indica rice. Rice seeds were de-husked manually and sterilized with 70% ethanol for 1 min followed by clorox for 15 min and then washing with autoclaved double distilled water for five times, aseptically in laminar air flow chamber. After, dried on sterile filter paper, the seeds were inoculated onto callus induction medium. The basic medium used for callus induction was N6 solid media with formulations (*i*) used were (1) 2.4 D 2 mg/L, (2) 2.4 D 3 mg/L BAP + 0.25 mg/L + Casein Hydrolisate 300 mg/L + Proline 600 mg/L, (3) 2.4 D 2 mg/L + Kinetin 0.5 mg/L + Casein Hydrolisate 500 mg/L + Proline 500 mg/L. As the carbon source used sucrose 30 g/L, the pH of media was adjusted to 5.8 before autoclaving, Phytagel 2.5 g/L, the autoclave for 20 minutes with a pressure of 17.5 psi and temperature of 121°C. At this step, 3 formulation mediums used to obtain embryogenic callus. The explants were then incubated for 21 d at ± 28°C in the dark condition. The number of embryogenic callus were then calculated.

b. Shoot Regeneration

Callus derivated from induction medium were used for regeneration. Media for shoot regeneration consists of four concentration levels of cytokinin BAP (*r*) and Kinetin (*k*) with a basic medium was MS + NAA 0.2 mg/L + Casein Hydrolisate 300 mg/L. The formulations were (1) BAP 1 mg/L + Kinetin 1 mg/L, (2) BAP 2 mg/L + Kinetin 1 mg/L, (3) BAP 1 mg/L + Kinetin 2 mg/L, (4) BAP 2 mg/L + Kinetin 2 mg/L. The mediums were added by 2.5 g/L phytagel (Sigma) and adjusted the pH to 5.8 before autoclaving. Each treatment consists of 4 repetitions. First, callus in regeneration media was incubated in the dark for 1 week at 28°C. After 1 week callus subculture on the same medium and incubated in light (16 h light / 8 h dark) at 28°C. At this step, the emergence rate of green spot callus and the formation of shoots variables were observed.

c. Statistical Analysis

Two experimental designs were used in this experimental works; non factorial randomized block design for the callus induction and a factorial randomized block design for shoots regeneration. Data were analyzed by Analysis of Variance (ANOVA) using SPSS. The significance differences were determined according to Duncan's multiple range test (DMRT). P values < 0.05 are considered to be significant.

RESULTS AND DISCUSSION

a. The Number of Embriogenic Callus

Embryogenic callus was observed after 21 in the culture media, in the dark condition at ± 28ºC. The categories of embryogenic callus were determined by the colour of callus. The embriogenic callus appears white to yellowish, shiny and friable (easily separated from other callus fragment), while the non embryogenic callus generally brown, rather pale, mushy watery and difficult to separate from the others [4]. The analysis results showed that the differences of the induction medium compositions were significantly effect for the induction of the number of embriogenic callus formations (Tab. 1).

Table 1. The development number of rice callus on different medium induction, after 21 days in culture.

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Treatment	Total Callus (%)	Embriogenic callus (%)	Non Embriogenic callus (%)
i_1	90.370 a	35.56 a	54.81 b
i_2	95.556 b	57.04 b	38.51 a
i_3	99.630 b	62.96 b	36.67 a

The number of followed by the same letter (notation), shows different at 5% level of DMRT analysis

The results showed that the percentage of embryogenic callus development in the additional of 2.4 D hormone (i_1) was lowest compared to that the development callus on the media i_2 and i_3, with the values of 35.56%, 57.04%, and 62.96% respectively. The differences in the ability of callus formation on i_1 media might be caused by the absence of cytokines hormones. In the time the equilibrium of both cytokinin and auxin in the media occurs, the division and elongation of cells would be maximized. However in some rice cultivars, the use of hormone auxin in particular 2.4 D only in the media was able to induce the callus, by the concentrations of 2 mg/L [5].

The explants source (embrio paddy) on the i_1 media were fail to perform the callus and its gradually turn to browning before transferred into regeneration media. These conditions lead to inhibited cell morphogenesis. Failure explants to perform the callus were probably caused of meristem damage during sterilization, and the differences of explants to sense and absorb nutrients in the media initiations, and plant growth regulators as well. Callus derived from explants on i_2 and i_3 mediums were higher performed compared to i_1. The additional of hormone cytokinin and amino acids in both media (i_2 and i_3) might be increased the number of explants in perform the callus, as well as the number of embryogenic callus. The use of the hormone auxin in the induction medium without addition of cytokines could be trigger the formation of callus, however the performance of callus were smaller and low frequency of embriogenic compared to that supported by both hormones.

Besides hormones, embryogenic callus formation was determined by the nitrogen content in the media. Amino acids are nitrogen organic sources that are quickly absorbed by the plants rather than the N-inorganic [6]. Casein hydrolysate (CH), tryptophan, yeast extract, and proline can improve the efficiency of embryogenic callus formation and plant regeneration from callus indica rice variety [7]. The addition of CH 500 mg/L and proline 500 mg/L in the callus induction medium (N6) enhances embryogenic callus formation up to 62.96% .

The addition of proline 100 mg/L on a medium containing 2,4D 3 mg/L can produce callus with a large diameter, yellowish color of callus, and friable [8]. Proline is the most effective supplement for callus formation. This amino acid functions as a precursor for the metabolism and cell division [9]. The formation of a green spot and the formation of shoots on a medium supplemented with proline were growth better than without proline [8].

b. The Emergence of Green Spot

The rate of emergence of green spot formation of the callus was determined in the regeneration media.In this experiment, this observation was restricted to 14 days after transferred into regeneration medium. Observation of this parameter was conducted by counting the day needed to established green spots on each aggregate callus.

The result showed that the composition of induction medium (*i*) was significantly effect on the emergence of the green spots (Tab. 2). The highest frequency of emergence green spot in the callus founds on the induction media i_2 following by i_3 dan i_1, with average 10.01, 11.18, and 11. 68 days, respectively. The 2.4D is a types of auxin which is the most widely used to induce callus in the plants cultures [10], including rice. The induction medium i_2 consists higher concentration of 2.4D (3 ppm), compared to i_1 and i_3 mediums that both used 2 ppm. The differences in emergence of green spot may be caused by differences in the composition of the culture media. The existence of the plant growth regulators and amino acids in the culture media were able to increase the emergence of green spots in the callus.

Table 2. The emergence rate of greeny callus

Treatment	Average (day)
i_1	11,68 b
i_2	10,01 a
i_3	11,18 ab

The number of followed by the same letter (notation) show different at 5% DMRT analysis

High concentration of auxin on the media can trigger somatic cells to induce genes that produce the necessary substances to complete a series of globular stage at somatic embryogenesis [11]. Moreover, the addition of growth regulator substances from the class of cytokines could increase proliferation of embryogenic callus (Tab. 3). The synthetic cytokine hormone, BA or BAP most widely used to induce the multiplication of shoots because it has a strong activity compared with kinetin [12]. BA or BAP has the same basic structure with kinetin but is more effective because it has a benzyl group [13].

The addition of amino acids on the induction media can support the growth and differentiation of callus. The use of CH of 300 ppm were significant effect on callus induction of rice by 74 % [14]. Other amino acid used is proline, which has an important role in being a trigger to bring up the somatic embryos, because it serves as a desiccation substance. The addition of the amino acid proline in the media can improve decrease the nitrogen content in the media, therefore the cells can improve embryo development to cotyledon stage, and eventually into plantlets [15]. The addition of the amino acid proline in the culture media such as creating the necessary stress conditions which will reduce water potential, increase the accumulation of nutrients in the cells and ultimately improve the development of the embryo [16]. Application of the amino acid proline is able to inhibit the plant tissue browning caused by oxidation of phenolic compounds generated from the oxidation of phenols in cultured cells [17].

Table 3. Interaction Factor (*i*) and Factor (*k*) on the emergence of Green Spot Rice Callus

Treatment	Average (day)
i_1k_1	12,396 b
i_1k_2	10,958 ab
i_2k_1	10,083 ab
i_2k_2	9,937 a
i_3k_1	10,937 ab
i_3k_2	11,417 ab

The number of followed by the same letter (notation) show different at 5% DMRT analysis

The regeneration callus from induction medium i_2 into media supplemented with 1 ppm (k_1) and 2 ppm (k_2) kinetin provides the highest results in forming green spots. It can be assumed that the embryogenic callus derived from the media i_2 produces the most optimal green spots callus. Moreover, the addition of hormone Kinetin on regeneration medium was significantly affect the rate of formation of green spots. Kinetin (6 - *furfurylaminopurine*) belongs to plant growth regulator of the class of cytokines that proven to increase cell division, proliferation and morphogenesis of shoots. This

result suggests that the addition of hormone cytokine such as kinetin in the regeneration mediums were highly induce the formation of a green spots on rice callus.

c. Emergence of Shoot Formation

The emergence of shoot formation was observed at the time shoots emergence from the callus and determined after the length were ± 0.5 cm. The result from this parameter showed that the compositions induction media (*i*) were significantly effect on the emergence of shoot formation (Tab. 4).

Table 4. Effect of Factor (*i*) on The Emergence of Shoot Formation

Treatment	Average (day)
i_1	55,82 b
i_2	53,08 a
i_3	55,78 b

The number of followed by the same letter (notation) show different at 5% DMRT analysis

The emergence shoots in medium (i_2) was faster than in the medium (i_3) and (i_1). The result also indicated that the callus derived from the induction media (i_2) combined with regeneration medium were produce buds faster compared to other treatments (Fig. 1).

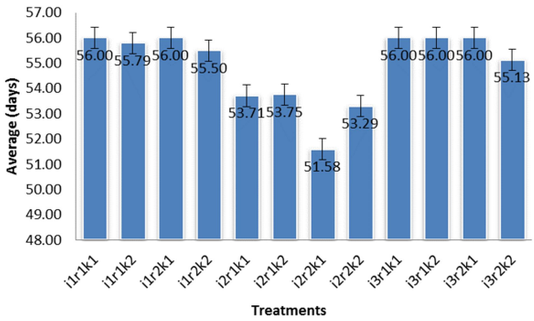

Fig. 1. The emergence rate of shoot formation on the combination of media compositions, induction media (i), concentration of BAP (r), and concentration of kinetin (k)

The callus from i_2 grown on regeneration medium r_2k_1 (BAP 2 ppm , 1 ppm Kinetin) showed rapidly increase bud formations, which appeared at 51.6 days after transfer. In the formation of green shoot regeneration, the optimum result was obtained in the treatment of r_2k_2. The result indicates that to induce the green spots in the callus and shoots required the kinetin in high concentrations, however to produce high formation of bud shoots required kinetin in lower concentrations. The number of shoots formation in the different media compositions were shown in Fig. 2.

Kinetin has role in promote cells morphogenesis. The process of cell elongations (G1 phase in cell growth) properly works in adequate of nutrients. The addition of kinetin on growth media may result in activation of RNA transcription and translation processes, to enter the phase of cell expansions (G2), furthermore into the phase of cell divisions [20]. The process of tissue growth in stadia culture is a gradual process of the cell as the smallest unit of a network that carrying the process related to life activities such as metabolism, growth, differentiation, and stimulation [21].

The faster of the formation of shoots, then it will accelerate regeneration to get new plants. The successfully of shoot growth depends on the source of explants, levels of nutrients, as well as the type and concentration of growth regulators [22]. The use of growth regulators in the medium depends on the direction of growth of the plant tissues that expected. To induce the formation of buds commonly used cytokinin, while for root formation or callus formation used auxin. However, sometimes it takes both of which depends on the ratio of cytokinin and auxin. The presence of one particular of growth regulators can enhance the activity of other endogenous growth regulator substances. The types and concentration of growth regulators for each plant tissues are not the same, the genotype and physiological condition influence the direction of the tissues development.

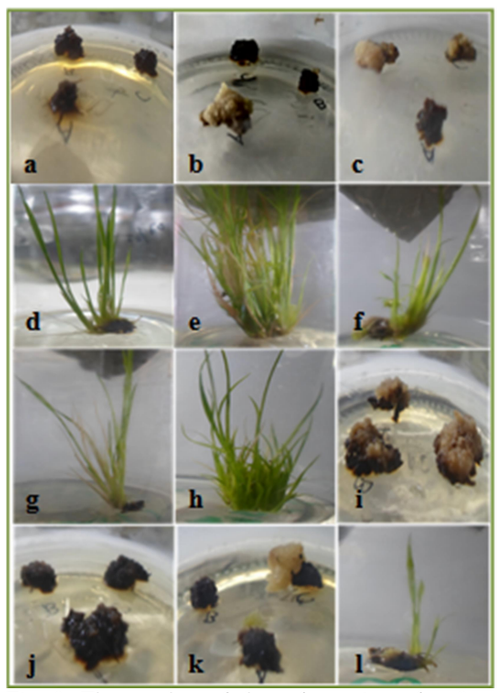

Fig. 2. The number of shoot formation after 8 weeks in the different media compositions. (a) i1r1k1, (b) i1r1k2, (c) i1r2k1, (d) i1r2k2, (e) i2r1k1 ,(f) i2r1k2, (g) i2r2k1, (h) i2r2k2, (i) i3r1k1, (j) i3r1k2, (k) i3r2k1, (l) i3r2k2.

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