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Duration for callus propagation of indica rice (*Oryza sativa* L.) cultivar Sangyod in suspension culture

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Abstract Calli of indica rice (*Oryza sativa* L.) cultivar Sangyod were propagated in a liquid culture medium containing 4.10 gL^{-1} modified Chu/Gamborg (NB) basal medium, 1 gL^{-1} L-proline, 2 mgL^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D), 1 mgL^{-1} 1-naphthalene acetic acid (NAA) and 30 gL^{-1} sucrose, maintained in shaker flasks (25±2 ℃, 100 rpm, under light for 27 days. Day 12 was found to be the best time for callus propagation, indicated by fresh mass (F.M.) doubling time (t_d) 11.73 day^{-1} and the highest cell density up to 66.83 gL^{-1} F.M. and 4.98 gL^{-1} dry mass, in the exponential phase. The productivity was 2.61 $gL^{-1}day^{-1}$ F.M. and yield of cell mass ($Y_{x/s}$) was 1.08 $g_{cell}g^{-1}_{glucose}$ F.M. The specific growth rate (μ) was 0.06 day^{-1} F.M. This data can be used to choose the optimum time for repeated subculturing callus to increase the productivity of high cell density cultures and it is an important for further study the physiological and morphological differentiation of cell suspension culture, improving plant regeneration systems.

Keywords: Callus propagation, Cell suspension culture, Exponential phase, Sangyod rice

Introduction

Rice is a widely consumed staple food from the grass *Oryza sativa*, commonly known as Asian rice. In Asian regions, ~90% of global rice is produced and consumed (Arnold, 1999) and it accounts for 23% of the world's total crop area. Global rice demand is projected to rise by 26% in the next 25 years and may reach nearly 555 million tons in 2035 (Song, 2003). In Thailand, rice is an important food crop. Thailand is a major rice producer and is a large rice exporter. Its rice exports rose from around 6.2 million tons in 1995 to 8.9 million tons in 2010 and grew at about 4% per year in 2005-2010 (International Rice Research Institute, 2013). Rice yield and quality are affected by diseases

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and environmental stresses, which may result to low productivity. The quantity of rice being produced is now insufficient for consumption (Poraha, 2015). Plant tissue culture can be used for developing high-quality clonal plants; it has the potential to provide rapid large-scale propagation of a particular plant and accelerate the production of new varieties of the plant (Anderson, 2017).

There are few studies regarding growing cell suspension culture of some rice varieties in Thailand, but there are no reports on suspension cultured cells of the Sangyod cultivar, an important commercial Thai cultivar. It was originally cultivated only in Phatthalung Province, in the south of Thailand (latitude: 7 ° 36' 33.59" N, longitude: 100 ° 04' 13.20" E) for a hundred years (Srinuttrakul *et al*., 2018). Since 1987, the Phatthalung Rice Research Center improved the Sangyod variety by pure line selection. The Sangyod rice is small, has a long-slender grain, dark red pericarp, soft and aromatic when cooked, delicious and nutritional. Sangyod rice is a good quality rice and is healthy, because it carries a large quantity of fibres and nutrients such as iron, vitamins, bioactive compounds, niacin, and has high antioxidant activity (Srisawat *et al*., 2010). Also, Sangyod rice has more protein and phosphorus than white rice, which has clear health benefits; it also contains antioxidants, oryzanol and γ-aminobutyric acid. Hence, this rice is very good for those looking for organic and healthy food. Currently, the demand for Sangyod rice is increasing globally and the price becaming higher than other local varieties. Thus, providing Sangyod rice for the market will benefit both farmers and consumers (Linh, 2017). However, the yield of this cultivar, compared with others, is low which makes it a threatened one. Manipulation through micropropagation techniques needs to be applied to address, both biotic and abiotic factors, that hinder the global production of rice in general, and can improve the yield of the existing cultivars.

We examined the growth rate of callus of indica rice cultivar Sangyod using cell suspension culture. A second goal was to investigate suitable time for repeated subculturing, based on the highest cells growth rate in cell suspension culture. This study may be useful in identifying better techniques for those who are responsible for planning high production of callus tissues and its subsequent plant regeneration.

Materials and methods

Prepraration of Sangyod rice callus

Mature seeds of Sangyod rice were used as the starting material for callus induction. First, the seeds were dehusked, washed with running tap water,

surface sterilized with 70% ethanol (v/v) for 1 min with gentle agitation, then steeped in 20% sodium hypochlorite (v/v) on the shaker (230 rpm, 30 min). After that, the dehusked seeds were washed three times with autoclaved distilled water. Finally, the disinfested seeds were blotted dry on an autoclaved coaster in a Petri dish to remove the excess water. The sterilized seeds were then cultured on a callus induction medium, identified by our previous study as the best medium for Sangyod rice callus induction (Meesook *et al*., 2019). It is the best solid medium for callus induction of Sangyod rice. The seeds were induced on NB medium supplemented with 2 mgL^{-1} 2,4-D, 1 mgL^{-1} NAA, 1 gL^{-1} L-proline, 30 gL^{-1} sucrose and 2.6 gL^{-1} phytagel. The pH was adjusted to 5.6-5.8 before autoclaving at 121°C. Then the seeds were incubated at 25±2°C in the dark. After 6 weeks of culture, callus was transferred to the suspension cell culture.

Measurement of the specific growth phase in Sangyod cell suspension

About 0.35 g calluses, obtained from the previous experiment, were excised and gentle grated in a 750 µm stainless steel sieve, then transferred into liquid medium with the same components as the callus induction medium. The cell suspension was maintained in shaker flasks (25±2 ℃, 100 rpm, continuous light). After culture, the three flasks of cell suspension cultured, at each of 0, 3, 6, 9, 12, 15, 18, 21, 24 and 27 days, were collected and filtered by an aspirator pump through a Whatman No. 1 filter paper for 1 min, then divided into cell suspension and liquid filtrate. For cells in the culture sample, we calculated fresh cell mass and dry cell mass (D.M.) for generating a cell growth curve for maximum callus induction. The fresh cell mass was calculated from the weight of filter paper + cell fresh residue (g) - the weight of filter paper (g). After that, the fresh cells were then dried in a hot air oven (110 ℃, 1 hour) and the mass determined from: the weight of filter paper + dried residue (g) - the weight of filter paper (g) (Poeiam and Saengdeuan, 2000). We calculated the specific growth rate (µ), the doubling time (t_d) and productivity (Godoy-Hernández and Vázquez-Flota, 2006; Farjaminezhad *et al*., 2013):

$$\mu = \frac{\ln X_t - \ln X_0}{t} \quad (1)$$

$$t_d = \frac{\ln 2}{\mu} \quad (2)$$

$$\text{productivity} = \frac{X_{max} - X_0}{t} \quad (3)$$

where X_0 is the initial cell density, X_t is the cell density at time, and X_{max} is the maximum cell density.

Total carbohydrates analysis

Glucose and total carbohydrate in the filtrate at the same times were measured by the phenol sulfuric method (Dubois *et al*., 1956). The filtrated was treated with 1 mL 5% phenol (w/v) and 5 mL 98% sulfuric acid (v/v). The mixture was incubated at room temperature for 20 min. Then, its absorbance at 490 nm was determined with a spectrophotometer, compared with glucose calibration curve to calculate X_t, the glucose concentration at time, t. The cell growth yield ($Y_{x/s}$) was calculated (Antimanon, 2015):

$$Y_{x/s} = \frac{X_{max} - X_0}{S - S_0} \quad (4)$$

Where S is the substrate at time of maximum cell density, and S_0 the initial substrate.

Statistical analysis

The experiment was designed with a completely randomized design (CRD). Three replicates of the treatments in days of suspension culture were made. The mean values of treatments were analysed in ANOVA, using IBM SPSS Statistics 23.0. Significant differences were determined using the Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$ level.

Results

We studied embryos of Sangyod rice cultured in NB medium supplemented with 2 mgL^{-1} 2,4-D, 1 mgL^{-1} NAA, 1 gL^{-1} L-proline, 30 gL^{-1} sucrose and 2.6 gL^{-1} phytagel. We observed that embryo proliferated from the scutellum region after 21 days of culture and the highest frequency of callus induction was seen after 6 weeks. The embryogenic callus morphology was recorded by scanning electron microscopy (Fig. 1A): the embryogenic callus was compact and light yellowish.

In cell suspension culture, using liquified NB medium for 27 days, during the first culture period (day 0) with initial friable calli (Fig. 1B), the calli density dramatically increased compared with any later days. Similarly, the cell mass increased in all treatments. No difference was observed in dry and fresh mass after 12, 15 and 18 days of culture (Table 1). For instance, the highest cell mass was seen at the 12^{th} days after cultivation (Fig. 1C) and the embryogenic calli had a light yellow color and a large and consistent size. There was a mass of unorganized cells from the initial friable callus.

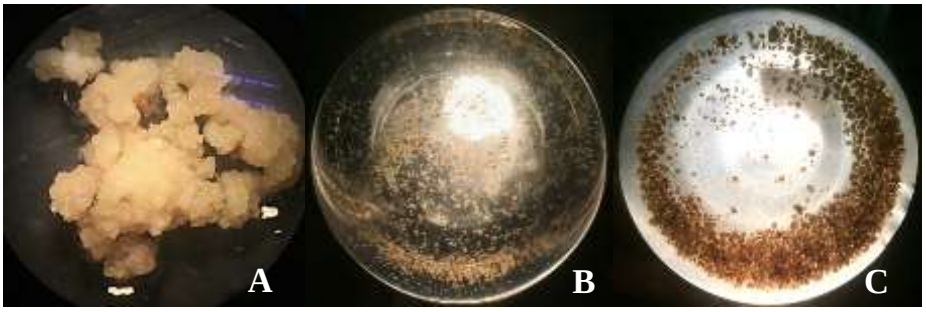

Figure 1. Callus formation of Sangyod rice in callus induction medium (A) SEM image showing proliferation of the scutellum leading to the callus. (B) 1st Day of cell suspension cultures. (C) Growing of cell after 12 days

Figure 2A shows that the callus growth curve had an exponential shape. The fresh weight was initially low during the first 3 days of culture. The cell suspension increased until 12 days (exponential phase). This period showed a significant increase in both total and dry cell mass. In particular, at the 12th day, the maximum cell masses were 67 gL^{-1} F.M. and 5.0 gL^{-1} D.M. After this period, callus growth was slow and no differences in dry mass were found from 15 to 27 days (stationary phase). The calli tended to turn brown with prolonged culture. Table 1 and Figure 2B show that the glucose concentration decreased exponentially over the 27 days of the experiment.

Table 1. Average of fresh cell mass, dry cell mass and concentration of glucose in cell suspension cultures of Sangyod rice for 27 days

Days of culture	Fresh cell mass (gL^{-1})/1	Dry cell mass (gL^{-1})/1	Glucose (gL^{-1})
0	35.48^{e}	2.88^{d}	31.46
3	40.93^{d}	3.38^{cd}	20.28
6	48.89^{c}	3.89^{bc}	11.30
9	60.37^{b}	4.80^{a}	6.58
12	66.83^{a}	4.98^{a}	2.49
15	66.10^{a}	4.73^{ab}	1.90
18	66.12^{a}	4.58^{ab}	1.80
21	63.44^{ab}	4.43^{ab}	1.10
24	63.76^{ab}	4.57^{ab}	0.84
27	63.04^{ab}	4.58^{ab}	0.27

[1]/In the same column, significant differences (determined the Duncan's Multiple Range Test at the $p \leq 0.05$ level) are indicated by different letters.

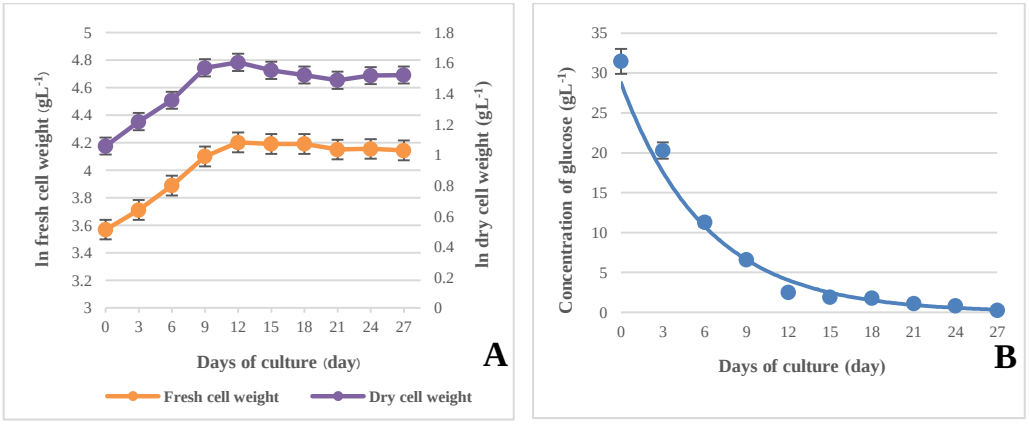

Figure 2. Relationship between days of cultivation and weight of cell mass (A) and concentration of glucose (B)

From cell densities versus time and the maximum cell density from dry cell mass and cell fresh mass were calculated using equations (1), (2) and (3). The specific growth rate (μ) was 0.06 day^{-1} in F.M., the doubling time (t_d) was 11.73 day^{-1} F.M. and productivity was in 2.61 $gL^{-1}day^{-1}$ F.M. The cell growth yield ($Y_{x/s}$) equation (4) was 0.07 $g_{cell}g^{-1}_{glucose}$.

Discussion

Results showed that the combination of NAA and 2,4-D was a better callus inductor than using single auxin (Trejo-Tapia *et al*., 2002; Din *et al*., 2016). A model of the callus growth curve is a key to determine the period for maximum callus and growth rate (Hussein *et al*., 2016; Linh, 2017). In general, the growth of cells in batch culture can be modeled with four different phases: lag, log or exponential, stationary and death (Fankhauser, 2015). During the log phase, the cells have high proliferation rates and good quality, such as large size, compact and light yellow color and well dispersed in the liquid medium. The quality of the callus in rice is one of the key factors to define the rate of regeneration (Amarasinghe, 2009; Linh, 2017). We observed that after a long period, the color of the callus turned to brown, but it they survived until the end of the culture period. Amalraj (2012) also suggested that the browning of callus may occur for various reasons under in vitro conditions. One possible reason is the accumulation of gases like CO_2, ethylene, acetaldehyde and so on, in the culture vessels. Ventilation would prevent this. Cytokinins have been reported

to induce ethylene production. The use of ethylene inhibitors like silver nitrate and silver thiosulphate can stop this. In addition, oxidation of phenolic compounds is the main factor in the browning of callus tissues. The most suitable medium for maintenance, shoot induction and somatic embryogenesis depends on the plant species. Quadram Institute Bioscience (2008) reported that the exponential phase is characterized by cell doubling. For this phase, plotting the natural logarithm of cell number against time produces a straight line. The slope of this line is the specific growth rate of the organism, which is a measure of the number of divisions per cell per unit time. However, the actual rate of growth relies on the growth conditions and morphology of plants (Sundram *et al*., 2012). Our finding confirmed previous reports that the growth curves for fresh and dry mass occurs in the first 12 days. In this study, the cells grew rapidly, i.e in an exponential phase, reaching a maximum fresh cell mass at 67 gL^{-1} and dry cell mass at 5.0 gL^{-1} at day 12. Thus, day 12 of culture is a suitable time for various applications. For instance, harvesting the cells for plant regeneration and repeated subculturing for cell growth. Besides, the cell mass is related to the glucose concentration and cell growth is reduced as the glucose concentration drops. We also observed that the cell suspension medium turned to brown and black as of the culture medium gets exhausted, the cells begin to die. By fitting the data into the model equations (1) to (3), the specific growth (μ) was 0.06 day^{-1}, the doubling time (t_d) was 11.7 day^{-1}, the yield ($Y_{x/s}$) was 1.08 $g_{cell}g^{-1}_{glucose}$ and the productivity was 2.61 $gL^{-1}day^{-1}$. With these parameters, cultivation can be more efficient and economical. Furthermore, our study detected no lag phase in the growth curve because the calli cultured from the solid medium had the same components as the liquid medium. Thus, cell suspension does not need to adapt to the new medium. Also, there was no death period because the plants grew slowly and death can be seen when the period of culturd was continued longer. This study contradicts the research of Poraha (2016), who investigated the cultivation of indica rice cultivar Khao Dawk Mali 105 on an NB medium containing 0.5 mgL^{-1} of 2,4-D and found that the cells grew from 0 to 15 days, with a lag phase, between days 0 to 12 and entered the exponential phase in days 12 to 15, with the highest growth shown on the 15th day. After that, the cell mass gradually stabilized, e.g. entered a stationary phase and then, entered a death phase. Right after the stationary phase, death started to happen which was caused by the inadequate nutrients in the medium

and accumulation of toxic substances released from the cells. In the future, our experiment should be compared with a new substrate to help identity, which substrate is capable of increasing the rate of growth of the Sangyod rice and the maximum yield, i.e. to generate a large number of sample cells in a short time and sufficient for work in various additional studies, such as plant regeneration, protoplast culture, cryopreservation and so on.

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