International Journal of Agricultural Technology 2015 Vol. 11(2): 279-305
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Callus Induction and Growing Cell Suspension Culture of Jow Haw Rice (*Oryza sativa* L.)

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Poraha, R., Poeaim, A., Pongjaroenkit, S. and Pongthongkam, P. (2015). Callus induction and growing cell suspension culture of jow haw rice (*Oryza sativa* L.*)*. International Journal of Agricultural Technology 11(2):297-305.

Abstract Mature embryos of rice (*Oryza sativa* L.) varieties Jow Haw was cultured on solid MS (Murashige and Skoog, 1962) and NB (Nitch and Nitch, 1969) medium supplemented with 0.5, 1, 2, 3 and 5 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D), 30 g/l sucrose, 1 g/l proline, 100 mg/l casein hydrolysate and 2.6 g/l phytagel. An optimum concentration of 3 mg/l 2,4-D in NB medium was found to be effective for callus induction which induced the biggest size of calli. Transferred the calli to liquid NB medium containing 3 mg/l 2,4-D, 30 g/l sucrose, 1 g/l proline and 100 mg/l casein hydrolysate. Study the growth phases at 0, 3, 6, 9, 12, 15, 18 and 21 days respectively, were determined by measuring fresh and dry weight of the cell. And viability of cell suspensions were determined by the method of fluorescein diacetate. The living cell of cell suspensions showed green fluorescence.

Keywords: Callus induction, Cell suspension, Growth curve, Jow Haw, RD6, Living cell

Introduction

Rice is the most important human food, eaten by more than half of the world's population every day. There are two types; glutinous and non-glutinous rice. In Asia where its covers half of the arable land used for agriculture in many countries (Cantrell and Hettel, 2004). Nevertheless, rice yield and quality are affected by pests and diseases, as well as by environmental stress. The quantity of rice has decreased which insufficient for consumption. Therefore, micropropagation of rice is considered as an important technique to produce sufficient food supplies. A success in micropropagation depends on the genotype of plant, type of the explants, composition and concentration of the basal salt and organic components and plant growth regulators in the culture medium (Ge *et al*., 2006). Amount of studies on increase efficiency of callus induction and cell suspension cultures have been reported in many rice

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cultivars. However, there is no report on the non-glutinous rice cultivar Jow Haw. Jow Haw is an upland rice and traditional rice found at the northern of Thailand. This grains shall possess the characteristics and size as follows: general characteristic is long grain, average length and width of the fell grain rice without breakage are not less than 7.0 mm. and 3.0 mm. Then, can resistance to blight diseases. When cooked, the grains' texture becomes tender. Therefore, the objective of this study was to found the suitable medium and concentration of plant growth regulators (2,4-D) for maximum callus induction and the biggest size of calli, growth curve of cell suspension cultures and viability for living cell from mature seeds of the rice cultivar Jow Haw.

Materials and methods

Surface sterilization

Jow Haw seeds were dehusked and surface sterilised in 70% ethanol for 1 min and then shaking in 20% of sodium hypochloride 30 min. After rinsing 3 times with sterile distilled water. Seeds were kept on sterilized filter paper in a Petri dish to remove excess water prior to transfer onto culture media.

Callus induction

The sterilised seeds were cultured on solid MS and NB media contained 0.5, 1, 2, 3 and 5 mg/l 2,4-D, 30 g/l sucrose, 1 g/l L-proline, 100 mg/l casein hydrolysate and 2.6 g/l phytagel. The pH of all media were adjusted to 5.8 before autoclaving at 121 ℃ for 15 min. The cultures were maintained at 25±1 ℃ under dark condition for 4 weeks. Each treatment consisted of 60 seeds.

The frequency of callus induction was counted after 4 weeks and calculated according to the following formula:

$$\text{Callus induction frequency (\%)} = \frac{\text{No.of seeds produced calli x 100}}{\text{No.of seeds cultured}}$$

And measure the size of callus by vernier calipers and calculated according to the following formula:

$$\text{Mean size of callus formation } (mm^3) = \frac{\Sigma \text{ (width x length x height) of calli}}{\text{No.of seeds that produced calli}}$$

Establishment of cell suspension cultures

Cell suspension cultures of Jow Haw rice was initiated from callus, 0.15 g of callus were transferred into tissue culture bottle 4 oz. containing 10 ml NB or MS liquid medium supplemented with 0.5, 1, 2, 3 and 5 mg/l 2,4-D that induced the biggest size of callus and combined 30 g/l sucrose, 1 g/l L-proline,

100 mg/l casein hydrolysate. The pH of all media were adjusted to 5.8 before autoclaved. The cultures were incubated on a rotary shaker at 120 rpm and maintained at 25±1 ℃.

Growth Measurements of cell suspension and cell viability

Cell suspension growth was study the growth phases at 0, 3, 6, 9, 12, 15, 18 and 21 days, respectively. Stable suspension cultures were used to demine the growth curve. The growth of the cell suspension cultures were determined by measuring fresh and dry weight of the cell every three days. Three replications were used for each experiment. The fresh weight of suspension cultures was measured by removing the medium. Cells were separated by filtration and washed with distilled water under vacuum. Dry weights of the fresh cells were determined after drying at 110 ℃ for 60 min, in a hot air oven until constant weight (Poeiam and Saengdeuan, 2000).

Study the viability of the cell suspensions with a fluorescent microscope. Suspension cells were transferred onto a microslide and stained with 0.5% fluorescein diacetate (FDA), covered with a cover slip. Under the fluorescent microscope, the living cell of cell suspensions showed green fluorescence.

Table 1. Callus induction frequency (%) and mean size of callus (mm^3) of Jow Haw rice which were cultured on solid MS and NB media supplemented with 0.5, 1, 2, 3, 5mg/l 2,4-D, 30g/l sucrose, 1g/l L-proline, 100mg/l casein hydrolysate and 2.6 g/l phytagel after 4 weeks.

Media	No. of seeds cultured	Concentration of 2,4-D (mg/l)	Frequency of callus induction (%)	Mean size of callus formation (mm^3)
MS	60	0.5	26.67	90.45
	60	1	45.00	84.64
	60	2	30.00	121.84
	60	3	38.33	79.39
	60	5	35.00	57.65
NB	60	0.5	27.00	112.02
	60	1	33.00	120.75
	60	2	25.00	135.99
	60	3	38.00	172.50
	60	5	35.00	114.81

Results and discussion

In present study the efficiency of MS and NB media containing different concentrations of 2,4-D was tested for callus induction in Jow Haw rice. MS and NB media are two well know media used to induce callus and regenerate plants in rice genotypes. The callus was induced successfully in all media and formed mostly embryogenic calli, which creamy, dry and compact appearance (Figure 1).

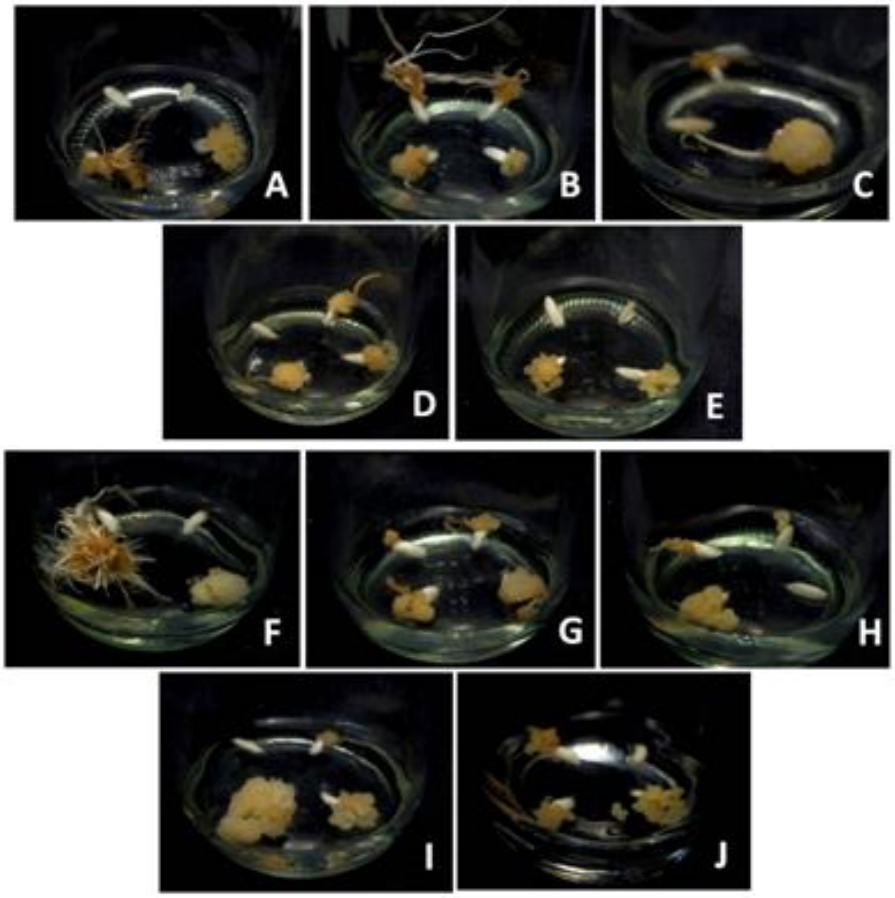

Figure 1. Callus from seeds of Jow Haw rice cultured on solid MS (A-E) and NB (F-J) media supplemented with 30 g/l sucrose, 1 g/l L-proline, 100 mg/l casein hydrolysate, 2.6 g/l phytagel and concentration of 2,4-D; (A, F) 0.5 mg/l 2,4-D, (B, G) 1 mg/l 2,4-D, (C, H) 2 mg/l 2,4-D, (D, I) 3 mg/l 2,4-D and (E, J) 5 mg/l 2,4-D after 4 weeks in culture.

The response of the seeds to different media and concentrations of 2,4-D for callus induction, the percentage of seeds producing calli were calculated for all media. That found the percentage of frequency of callus induction were not much difference between MS and NB medium. However, the formation of callus was observed, suggesting that 2,4-D play a crucial role in callus induction of rice as described by Chen *et al*. (1974), Maeda (1980) and Bajaj (1991).

Callus was initiated from seeds after two weeks of cultivation. The proliferation of callus was continued until the fourth weeks, Then the frequency of callus induction and size of callus were determined. The results are show in Table 1. The biggest size of callus was 172.50 mm^3 on NB medium containing 3 mg/l 2,4-D, followed by 2 mg/l (135.99 mm^3), 1 mg/l (120.75 mm^3), 5 mg/l (114.81 mm^3) and lowest 0.5 mg/l 2,4-D (112.02 mm^3). The 0.5 mg/l 2,4-D supplement developed shoot and root more than other 2,4-D concentrations as seen in Carsomo and Yoshida (2006). Mean size of callus formation, which cultured on NB medium was found bigger than MS medium. The difference in the composition of culture medium can result in variation in callus induction (Torbet *et al.*, 1998). NB medium composed of nitrogen sources as N_6 medium and PP medium (Poonsapaya *et al.*, 1989) as recommended by Rueb *et al.* (1994). It is, perhaps due to the reason that NB medium contained more nitrogen than the MS medium. However, Raina (1989) reported that 2,4-D is the most suitable auxin for callus induction of rice in tissue culture, although the optimum concentration of 2,4-D varied depending on the explants source and genotype. Results of the present study were in agreement with those of Visarada *et al*. (2002) cultured four varieties of rice; Seshu, Nagarjuna, Rasi, and Jaya for callus induction on NB medium containing 3 mg/l 2,4-D found that percentage of callus induction were highest when compared to other concentrations of 2,4-D (57.1, 81.4, 94.1 and 78.1 %, respectively). Tariq *et al*. (2008) reported Fakhre Malakand rice gave highest mean weight (0.26 g) on N_6 medium containing 3 mg/l 2,4-D. In case of Basmati 370, the callus induction frequency was increased by increasing the concentration of 2,4-D from 1 to 2 mg/l and was maximum at 3 mg/l (Rsahid *et al*., 2003)

Table 2. The measurement of fresh and dry weight for cell suspension cultures of Jow Haw rice in liquid NB medium supplemented with 3 mg/l 2,4-D, 1 g/l L-proline, 100 mg/l casein hydrolysate and 30 g/l sucrose for 21 days.

Days	Fresh weight (g/10 ml)	Dry weight (g/10 ml)
0	0.1767	0.0059
3	0.2923	0.0066
6	0.2956	0.0083
9	0.2961	0.0117
12	0.3345	0.0169
15	0.3407	0.0200
18	0.2889	0.0122
21	0.2881	0.0095

Calli cultured ineitiated from embryogenic callus was cultured in liquid NB medium supplement with 3 mg/l 2,4-D. Growth rate was measured by fresh and dry weight at 3 days intervals, an exponential growth phase of cell suspension within the lag phase in the range of 0-3 days, 6-12 days during the log phase, stationary phase during the period of 12-15 days, and the period of the death phase after 15 days, which were presented in Figure 2(A-B). After 15 days, calli were decreasing and browning Bushra *et al.* (2009) reported that the browning subsequent death of explants and cultures could be attributed to the oxidation of polyphenols. The cell suspension in the log phase, that fast growth. Therefor, suitable to be used in activities such as transfer into fresh medium or used for regeneration. In this reported of Lima *et al*. (2008) reported that growth deceleration occurs as a result of the usage of nutrients and the accumulation of toxic substances in the culture medium. It is appropriate to subculture the callus at the beginning of this phase. Which found the maximum growth rate fresh and dry weight cell in the suspensions culture was increase rapidly approximately 12-15 days of culture. On 15th, the maximum of fresh weight and dry weight were 0.3407 and 0.0200 g per 10 ml of media, respectively (Table 2). The suspension cultured embryogenic calli showed form, compact, yellowish, nodular, easy dispersion and fast growth of cells in liquid medium in Figure 3A and Figure3B showed cell suspension scanning from the brightfield microscope for 21 days. Then, study the characteristics of cell suspension under microscope fluorescence at wavelength 440-480 nm. The live cell and dead cell in medium were observed the living cells of cell suspensions showed green fluorescence but death cells were not stain after staining 0.5% fluorescein diacetate (FDA) in figure 3C.

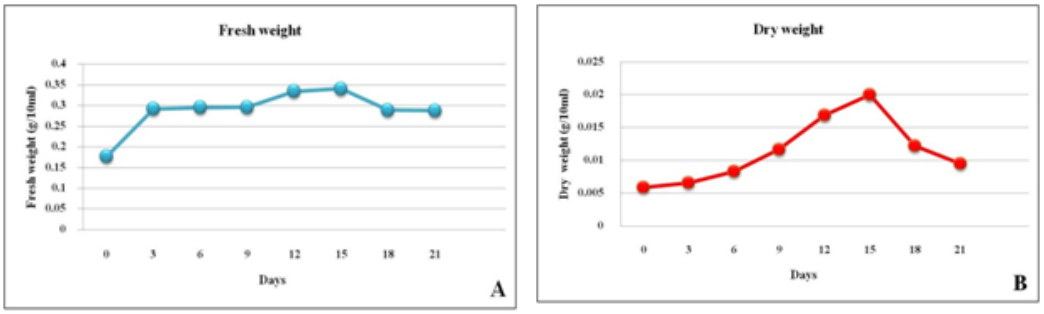

Figure 2. Growth rate of fresh (A) and dry (B) weight cell for suspension of Jow Haw rice in liquid NB medium contained with 3 mg/l 2,4-D, 1 g/l L-proline, 100 mg/l casein hydrolysate and 30 g/l sucrose for 21 days.

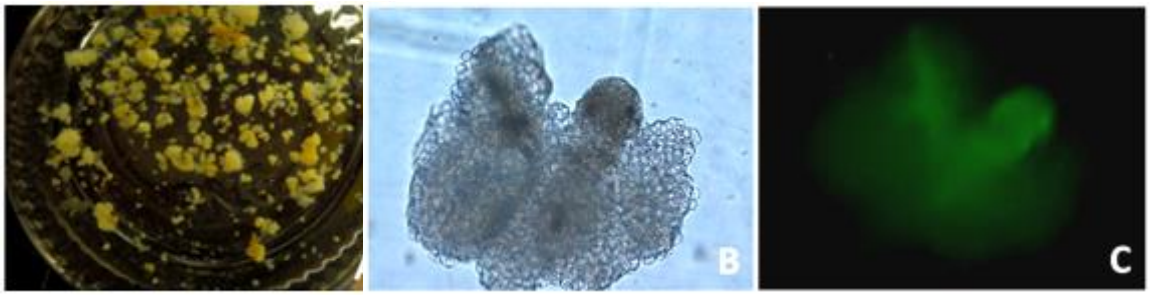

Figure 3. (A) Cell suspension cultured on liquid NB media supplemented with 3 mg/l 2,4-D, 1 g/l L-proline, 100 mg/l casein hydrolysate and 30 g/l sucrose. (B) Cell suspension scanning from the brightfield microscope for 21 days. (C) Viability of suspension cells was determined by fluorescein diacetate.

Conclusion

The current study indicates that the NB medium supplemented with 3 mg/l 2, 4-D gave the biggest size callus (172.50 mm^3) from matured seeds of Jow Haw rice**.** Cell suspension cultures in 10 ml liquid NB medium supplemented with 2,4-D concentration of 2 mg/l, were found the highest of fresh weight and dry weight were 0.3407 and 0.0200 g/10 ml of media, respectively. The maximum growth rate is after 12-15 days of culture. The living cell of cell suspension showed green fluorescence. The method presented here will be useful in biotechnological approaches to improve Jow Haw rice (*Oryza sativa* L.) through in vitro induced artificial mutations using radiation

such as gamma-rays, for improving this world's staple food crop either to increase yield or to improve nutritional quality.

Acknowledgement

This research paper was supported by the Office of the Higher Education Commission Thailand and Department of Biotechnology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.

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