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Somatic Embryos Induction of East Kalimantan Local Rice (*Oryza sativa* L.) Cultivars and In Vitro Selection Against Salinity

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ABSTRACT

Soil salinity is one major environmental constraint on rice production, especially in coastal areas. The development of salt-tolerant genotypes is considered to be the most effective breeding strategy to overcome the constraint. This study aims to induce somatic embryos formation of East Kalimantan local rice cultivars and to obtain tolerant somatic embryos under saline condition via in vitro selection. Four commonly cultivated local rice cultivars, namely Buyung, Siam, Ketalun Tawar and Serai Gunung, were used in this study. The somatic embryos were produced using three different plant growth regulator (PGR) compositions. The salinity tolerance level of somatic embryos was induced by in vitro selection in salt toxicity medium containing 0 mM; 50 mM; 100 mM; 150 mM; 200 mM NaCl. The best medium for somatic embryogenesis contains 1 mg/l 2,4-D + 0.5 m/l BAP, resulting the highest percentage of cream and white non-compact callus on the tested cultivars. More than 70% of the somatic embryos were tolerant against salinity (NaCl 200 mM). However, only somatic embryos derived from Serai Gunung could regenerate into normal plantlets.

INTRODUCTION

Rice is the most widely cultivated crop and serves as a staple food for more than half of world population. The need for rice increases, along with the population growth. However, efforts to increase rice production in order to be self-sufficient have faced obstacles due to limited arable land. Many agricultural lands have shifted their functions to non-agricultural purposes. One promising alternative to expand agricultural land is by utilizing marginal land, such as saline soils (Tedeschi, 2020).

Saline soil contains salts that can dissolve in large quantities. More than 800 million hectares of agricultural land in the world has been affected by salt (FAO, 2020). Salinity problems occur under all climatic conditions in arable and non-arable soils, in irrigated or dry land agriculture. Soil salinity is an agricultural constraint in over 100 countries in all continents (Shahid, Zaman, & Heng, 2018). Therefore, salinity becomes an important issue in the development of agriculture nowadays.

Most of crop species are sensitive to salinity, including rice (Grieve, Grattan, & Maas, 2012). High soluble NaCl in root zone affects plant growth through salt toxicity due to excessive absorption, decreasing water absorption, osmosis pressure, nutrient imbalance, and limited major nutrient uptake including nitrogen (FAO, 2005; Nadeem et al., 2020). In addition, soil containing high amount of Na^+ will reduce the availability of Ca^+, Mg^{2+}, and K^+ and the absorption of P (Machado & Serralheiro, 2017; Mohammadi, Rahimpour, Pirasteh-Anosheh & Race, 2022), which will cause disruption of several enzymatic reactions that are important for plant growth and yield (Wakeel, 2013). The increase in Cl^- is usually followed by a reduction in the content of NO_3^- in plant canopy (Guo, Zhou, Li, Yu, & Luo, 2017). Therefore, saline land has not been widely used for crop cultivation, since the toxic and root osmotic pressure effects, can result in the disruption of the plant growth (Slinger & Tenison, 2005). In addition, saline soil is difficult to manage with conventional treatments; therefore, the most

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effective way for optimizing the use of saline soil for agricultural land is using tolerant varieties (Litalien & Zeeb, 2020).

Somatic embryogenesis and in vitro selection are tissue culture based techniques which have been proven as an effective and efficient method towards new improved traits on crop plants (Kumar, Gill, & Gosal, 2018). Somatic embryogenesis is a process whereby embryos develop from somatic cells or tissues (von Arnold, Sabala, Bozhkov, Dyachok, & Filonova, 2002). For mass propagation, somatic embryos have benefits due to the high multiplication rate, easily propagated in liquid media, the handling of large numbers of embryos at once (Agisimanto, Normah, & Ibrahim, 2019; Loyola-Vargas & Ochoa-Alejo, 2016). Therefore, somatic embryos are potential explants for plant developmental purposes, including in vitro selection for developing stress-tolerant plants. In vitro selection has been successfully applied for obtaining tolerant lines against several abiotic stresses (Amrita, Sunita, & Ranjan, 2015; Rai, Kalia, Singh, Gangola, & Dhawan, 2011; Wani, Sofi, Gosal, & Singh, 2010). The tolerance can be acquired by applying the corresponding selecting agents, such as NaCl for salt tolerance. The intensity of selection can be strengthened to increase the frequency of variants (Biswas, Chowdhury, Bhattacharya, & Mandal, 2002). In vitro selection for inducing salinity stress tolerance has never been applied to the East Kalimantan rice population. Therefore, this study was carried out to increase the tolerance of local rice cultivars from East Kalimantan against salinity through in vitro selection technique.

MATERIALS AND METHODS

This study was conducted from October 2017 to March 2018, in Laboratory of Biotechnology, Faculty of Agriculture, Mulawarman University, Samarinda, East Kalimantan, Indonesia.

Somatic Embryos Induction

This experiment used a completely randomized design with two factors and ten replications. The first factor was East Kalimantan local rice cultivars, namely: Buyung, Siam, Ketalun Tawar, and Serai Gunung. The second factor was somatic embryo induction media, i.e., Murashige and Skoog (MS) solid media containing 30 g/l sucrose with three different PGR compositions, i.e.: 1 mg/l 2,4-D + 0.5 mg/l BAP (M_1), 2 mg/l 2,4-D + 0.5 mg/l BAP (M_2) and 3 mg/l 2,4-D + 0.5 mg/l BAP (M_3).

Mature rice seeds were used as explants in this study. The selected seeds were washed using detergent and rinsed with distilled water. The lemma and palea of the seeds were manually removed. The caryopsis were immersed in 70% ethanol for 1-2 minutes, then consecutively followed with 30% for 15 minutes and 10% for 5 minutes of NaClO. Prior to inoculation, explants were rinsed twice for 3-5 minutes using sterile distilled water, then drained on sterile filter paper. Explants were inoculated on the somatic embryos induction media based on the arranged treatments.

In Vitro Selection of Somatic Embryos against Salinity

The somatic embryos derived from the best induction media treatment were furtherly used for in vitro selection against salinity. In vitro selection was performed by incubating one month-somatic embryos into liquid selection media. A completely randomized design with two factors and five replications was used for this experiment. The first factor was somatic embryos of four local rice cultivars of East Kalimantan, Buyung, Siam, Ketalun Tawar, and Serai Gunung. The second factor was salinity selection media containing at 0 mM, 50 mM, 100 mM, 150 mM, and 200 mM.

Salinity selection media was prepared by dissolving NaCl in liquid MS medium containing 30 g/l sucrose and supplemented with the same growth regulators where the embryos were obtained. To prevent the explants from sinking, the explants were placed on a raft in the form of a piece of foam (0.75 cm high) covered with filter paper which was placed at the bottom of the bottle. The 0.5 cm callus clump was placed on the filter paper and incubated for a month in the salinity selection media. The cultures were maintained under light conditions (24-hour photoperiod) at 25±2^{o}C.

Callus Regeneration

Callus regeneration was carried out using solid MS medium containing 30 g/l sucrose supplemented with 1 mg/l BAP. The regeneration induction was conducted under light conditions (24-hour photoperiod) at 25±2^{o}C.

Data Collection and Analysis

The percentage of callus development was carried out after 30 days incubation. The callus quality was also observed from its structure and color. The percentage of survived callus was recorded after the embryos one-month-old in the salinity selection media. The regeneration ability of callus was observed by calculating the number of calli forming roots or shoots in the regeneration media. Percentage data of explants forming callus and callus survival on salinity selection media were analyzed using analysis of variance, and continued by DMRT with 95% level of significance.

RESULTS AND DISCUSSION

Somatic Embryogenesis of East Kalimantan Rice Cultivars

Somatic embryogenesis and organogenesis can be either enhanced or inhibited by phytohormones. In this study, the characteristics of somatic embryos induced in the East Kalimantan local rice cultivars were observed from structures (Fig. 1) and colors of callus (Fig. 2). The best medium to induce somatic embryos was the media containing 1 mg/l 2,4-D + 0.5 mg/l BAP. This medium was able to induce the highest percentage of the non-compact callus structure (Fig. 1B) and produce cream color (Fig. 2A) on the four local upland rice cultivars (Table 1).

Callus structure is one of markers to assess the callus growth. A compact callus structure usually represents secondary metabolites producers since it accumulate more secondary metabolites than non-compact callus structures (Lindsey & Yeoman, 1985). The secondary metabolite production calli generally homogenous, *dedifferentiated* cells, and non-embryogenic calli (Efferth, 2019). However, non-compact callus structures experience faster cell division than compact structures. The compact callus structure has a slow cell division rate. Compact callus has elongated cells and overlap each other, forming callus clump, and have the potential to develop toward organogenesis and have no potential to become somatic embryos (Rahmah, Anwar, & Swasti, 2020).

The non-compact/friable callus was higher than the compact structure in all cultivars and in all somatic embryos induction media. The non-compact callus structure is embryogenic potential. There are many embryonic developments that can regenerate into plants, in this structure (Armstrong & Green, 1985). In addition, friable callus also facilitates the separation into single cells in suspension culture and the callus multiplication is easier to handle. The friable callus appearance is due to the large intercellular spaces leading to the increase of oxygen aeration between the cells.

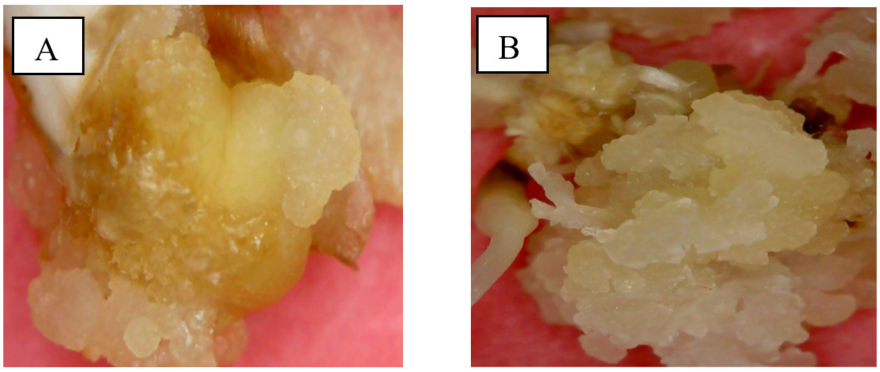

Fig. 1. Callus structures of the local rice cultivars; (A) compact, (B) Non-compact

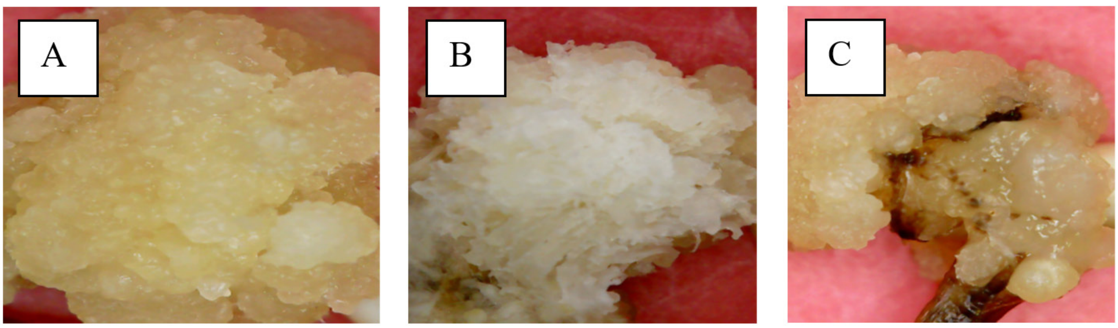

Fig. 2. Variation of callus color of the local rice cultivars; (A) Cream (yellowish white), (B) White, (C) Brownish

Table 1. Percentage of non-compact structure and creamish color of callus at 30 days after inoculation

Treatment		Non- Compact Callus Structure (%)	Creamish Callus Colour (%)
Local rice cultivars	Media		
Buyung	1 mg/l 2,4-D + 0.5 mg/l BAP	100.00 (35/35)*	88.57 (31/35) *
	2 mg/l 2,4-D + 0.5 mg/l BAP	100.00 (33/33)	96.97(32/33)
	3 mg/l 2,4-D + 0.5 mg/l BAP	100.00 (36/36)	97.22 (35/36)
Siam	1 mg/l 2,4-D + 0.5 mg/l BAP	100.00 (38/38)	100.00 (38/38)
	2 mg/l 2,4-D + 0.5 mg/l BAP	94.59 (35/37)	91.89 (32/37)
	3 mg/l 2,4-D + 0.5 mg/l BAP	100.00 (38/38)	100.00 (38/38)
Ketalun tawar	1 mg/l 2,4-D + 0.5 mg/l BAP	100.00 (39/39)	94.87 (37/39)
	2 mg/l 2,4-D + 0.5 mg/l BAP	100.00 (39/39)	100.00 (39/39)
	3 mg/l 2,4-D + 0.5 mg/l BAP	100.00 (39/39)	97.44 (38/39)
Serai Gunung	1 mg/l 2,4-D + 0.5 mg/l BAP	100.00 (28/28)	71.43 (20/28)
	2 mg/l 2,4-D + 0.5 mg/l BAP	100.00 (35/35)	100.00 (35/35)
	3 mg/l 2,4-D + 0.5 mg/l BAP	97.37(37/38)	92.11 (35/38)

Remarks: *x/y; x = number of non-compact callus structure or callus creamish color; y = number of explants forming callus

Table 2. Percentage of explants forming callus of local rice cultivars on the different combination of PGR concentration (%)

Local rice cultivars	PGR Concentrations			Average
	1 mg/l 2,4-D + 0.5 mg/l BAP	2 mg/l 2,4-D + 0.5 mg/l BAP	3 mg/l 2,4-D + 0.5 mg/l BAP	
Buyung	87.50 aB	82.50 aA	90.00 aA	86.67 A
Siam	95.00 aB	92.50 aAB	95.00 aA	94.17 A
Ketalun Tawar	97.50 aB	97.50 aB	97.50 aA	97.50 B
Serai Gunung	70.00 aA	87.50 bAB	95.00 bA	84.17 A
Average	87.50 a	90.00 a	94.38 a	

Remarks: The means followed by the same lowercase letter in the same row and the same capital letters in the same column were not significantly different at the 5% of DMRT test.

Table 3. Percentage of callus survival of local rice cultivars after a month on the different concentration of salinity media

Local rice cultivars^{ns}	Salinity Concentration (NaCl)^{ns}					Average
	0	50 mM	100 mM	150 mM	200 mM	
Buyung	100.00	90.00	100.00	90.00	70.00	90.00
Siam	85.00	100.00	95.00	80.00	85.00	89.00
Ketalun Tawar	90.00	80.00	90.00	85.00	90.00	87.00
Serai Gunung	80.00	85.00	95.00	80.00	80.00	81.00
Average	88.75	88.75	95.00	83.75	81.25	

Remarks: ^{ns} means that all treatments did not significant based on salinity concentrations on anova test at α = 5%.

Callus color as the visual appearance of callus illustrates the level of callus development. In the induction media, Buyung, Siam, KetalunTawar, and Serai Gunung cultivar are dominated by cream and white callus, indicating active growing cells. The brownish callus indicated that the cells were less active and physiologically decrease in growth (Fig. 2). According to He et al. (2009) the brownish less active cells usually also synthesizes, excretes and accumulate a high amount of phenolic compound based on histochemical analysis.

The local rice genotypes significantly affected the somatic embryogenesis rate (Table 2). Ketalun Tawar had the highest explant forming callus (97.50%) and was significantly different from Buyung, Siam, and Serai Gunung. An interaction between rice genotype and callus induction medium was also observed in the percentage of explants forming callus. The highest callus developmental rate of each genotype varied in different callus induction media, showing the influence of plant growth regulator in callus induction (Liu et al., 2021). The somatic embryos induction media showed significantly different effect only in Serai Gunung cultivar, in which the increase of 2,4 D concentrations on 2 mg/l 2,4-D + 0.5 mg/l BAP and 3 mg/l 2,4-D + 0.5 mg/l BAP media leads to an increase of somatic embryogenesis rate.

The percentage of explants forming callus ranged from 84.17 to 97.50% in all treatment combinations. This result was higher than the callus formation rate of Ciherang, Cisadane, IR64, and Taipe 309 as reported by Purnamaningsih (2006) which only reached ca. 31-70%. Callus formation and growth in rice were affected by the presence of light during incubation. In our study, the dark conditions during incubation merely induced callus formation. A dark condition might inhibit the growth of gametic embryos in the rice seeds (explant) due to the absence of the light since the photosynthetic process does not occur. Thus, the nutrition transportation from the rice endosperm to the embryo is increased through the scutellar layer (Sato, 2008), where the callus is induced to grow (Nurhasanah, Ramitha, Supriyanto, & Sunaryo, 2018).

In Vitro Selection of Somatic Embryos Against Salinity

The explants used for in vitro selection are embryogenic calli cells and these were selected to have friable texture with cream to white color. The degree of tolerance of embryonic callus was observed after one month incubation in media treatments (liquid MS + 1 mg/l 2,4-D + 0.5 mg/l BAP+ NaCl treatment). There was no significant difference among the genotypes, and salinity stress concentration, as well as their interaction on the percentage of callus survival (Table 3). The callus allegedly quite tolerant at 100 mM NaCl; however, the tolerant tends to decrease in a higher NaCl concentrations (Fig. 3). The survived callus had cream or white color or turned into green in saline conditions. In some cases, the callus color turned into brown or black, indicating the cells were undergoing physiological decrease and dead. The appearance of browning color might also be due to the imbalance of nutrient within the media that induced formation of quinones in callus and the oxidation process of polyphenols (Hussain, Naz, Nazir, & Shinwari, 2011).

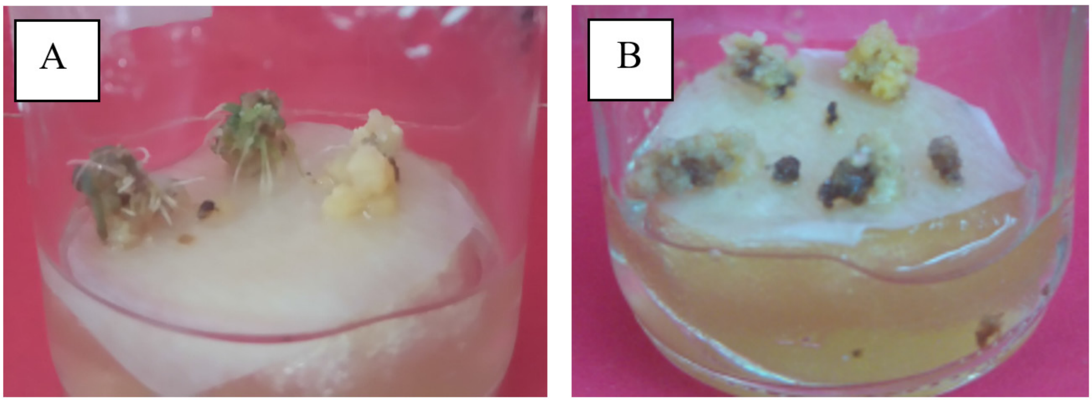

Fig. 3. Callus performance in salinity stress condition: (A) tolerant callus with new proliferated cell mass, (B) salinity sensitive callus showing physiologically decline cell

Callus Regeneration

The saline tolerant somatic embryos were induced to form complete plantlets in regeneration media. Plantlets regeneration was observed only in embryonic callus from Serai Gunung. There was no adequate root or shoot growth from the subsequent embryonic callus development of Buyung, Siam and Ketalun Tawar in regeneration media (Table 4). Six plantlets were regenerated from somatic embryos derived from MS with 50 mM and 200 mM NaCl media. In our previous study, Serai Gunung was also observed as the most responsive genotype to anther culture compared to other East Kalimantan local upland rice cultivars including Buyung (Nurhasanah, Pratama, & Sunaryo, 2016).

In some accessions, the callus formed roots without the appearance of visible shoot (Fig. 4A). Serai Gunung was observed to have more roots, followed by Buyung and KetalunTawar (Table 5). Aside root and shoot formations, callus was also developed into creamy secondary somatic embryos (Fig. 4B). However, most of them were undifferentiated, then turning into brown and finally, died (Fig. 4C).

Callus structure described the regeneration ability to form shoots or roots. The globular callus with many nodules and transparent colors usually has higher potential to form buds than the compact with brownish color callus (Armstrong & Green, 1985). The most important factor affecting the regeneration ability of callus are genotype and media or the PGR (Khan, Ahmed, Shahzadi, & Shah, 2015). Plant growth regulators play an essential role in regulating biological processes in plant tissues. The balance of nutrients in growing media greatly affects the growth of callus and direction of differentiation to form other organs (Ikeuchi, Sugimoto, & Iwase, 2013). Auxins and cytokinins may interact through synergistic, antagonistic and additive mechanism depending on the culture condition of the inoculated tissue that drive the developmental direction towards callus or shoot formation (Mohd Din et al., 2016).

In the process of organ formation, complex interactions between exogenous and endogenous growth regulators might be happened. Gatphoh et al. (2018) reported that maximum multiple shoot regeneration was obtained in MS medium containing higher cytokine concentration, 5 mg/l BAP + 0.05 mg/l NAA, compared to the current study. Similar results were also found in Kamal, Nasuruddin, Haque, & Yasmin (2013) and Sankepally & Singh (2016). On the other hand, Wijesekera, Iqbal, & Bandara (2007) observed that roots were regenerated on the free hormone regeneration medium.

Table 4. Callus forming shoots of local rice cultivars on the different salinity concentration media*

Local rice cultivars	Salinity Concentration (NaCl)				
	0	50 mM	100 mM	150 mM	200 mM
Buyung	0/20	0/20	0/20	0/20	0/20
Siam	0/20	0/20	0/20	0/20	0/20
Ketalun Tawar	0/20	0/20	0/20	0/20	0/20
Serai Gunung	0/20	1/20 (5)	0/20	0/20	1/20 (1)

Remarks: * Data is presented in the form of x/y or x/y (z); x = number of callus forming shoots, y = number of callus regenerated, z = number of shoots/plantlets.

Table 5. Callus forming roots of local rice cultivars on the different salinity concentration media*

Local rice cultivars	Salinity Concentration				
	0 mM	50 mM	100 mM	150 mM	200 mM
Buyung	2/20	0/20	1/20	0/20	4/20
Siam	0/20	0/20	0/20	0/20	0/20
Ketalun Tawar	1/20	0/20	0/20	1/20	1/20
Serai Gunung	5/20	1/20	0/20	2/20	3/20

Remarks: * Data is presented in the form of x/y; x = number of callus forming roots, y = number of callus regenerated.

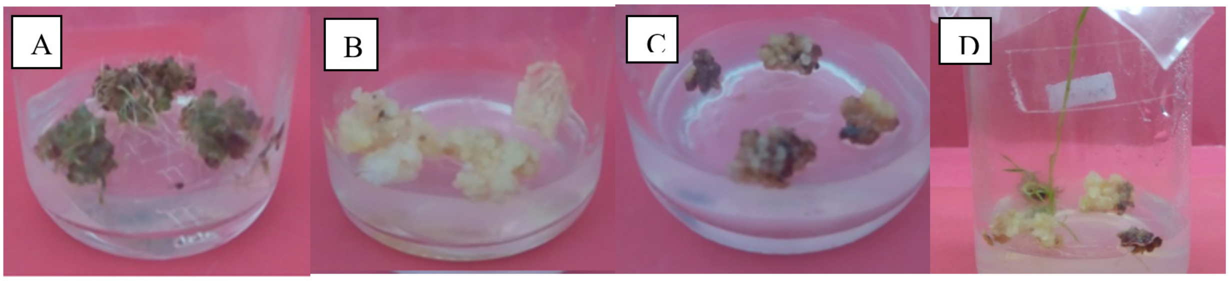

Fig. 4. Callus regeneration: (A) callus forming roots; (B) callus forming secondary embryonic callus; (C) callus degeneration turned into brownish or blackish; (D) callus forming shoots/plantlets

CONCLUSION

Among the evaluated media, MS containing 1 mg/l 2,4-D + 0.5 mg/l BAP gave the highest percentage of non-compact callus structure with cream and white color on all tested local rice cultivars. More than 70% of somatic embryos were tolerant to saline conditions up to the NaCl concentration of 200 mM. Among the local rice cultivars tested, only Serai Gunung callus could develop into plantlets in regeneration media.

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