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PLANT REGENERATION OF SUGARCANE (*Saccharum officinarum* L.) CALLI *IN VITRO* AND ITS RESPONSE TO GAMMA IRRADIATION

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ABSTRACT

One way of mutation breeding in sugarcane is to expose *in vitro* growing calli to gamma ray, regenerate the calli into plants, and then evaluate the plants . This research sought to study *in vitro* plant regeneration of sugarcane (*Saccharum officinarum* L.) calli and to determine LD_{50} of gamma ray for irradiation of embryogenic calli. The research was conducted at The Plant Laboratory, The University of Lampung, Lampung, Indonesia in 2014. Leafrolls of sugarcane were cultured on callus-inducing (CI) medium containing MS salts, 30 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol, 150 m L^{-1} coconut water, 0.1 mg L^{-1} thiamine-HCl, 0.5 mg L^{-1} pyridoxine-HCl, 0.5 mg L^{-1} nicotinic acid, 2 mg L^{-1} glycine, and 3 mg L^{-1} 2,4-D. The embryogenic calli were cultured on shoot-inducing (SI) medium which was the same as the CI medium, except that the SI medium used 2.5 mg L^{-1} benzyladenine and no coconut water. Shoots were rooted on root-inducing (RI) medium containing different concentrations of indolebutyric acid (IBA) (0, 2, 5, 7.5, 10 mg L^{-1}). The RI medium was the same as the SI medium except for the plant growth regulators. The most effective IBA concentration for rooting of shoots was 5 mgL^{-1} . Plantlets with highest number of roots showed highest survival rate (68.4%). A radio sensitivity study by irradiating embryogenic calli with gamma ray (0, 5, 10, 15, 20, 25, 30, 40, 50, 60 Gy) showed that the LD_{50} was 17 Gy. The irradiated calli were successfully regenerated into plantlets and acclimatized to external environment. Results of these studies could be very useful for mutation breeding of sugarcane.

Key words: 2,4-D, calli, IBA, LD_{50}, mutation

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is one of the major commercial estate crops, which is the biggest source of sugar, contributing to 70% of sugar consumption in the world. As the world population increases, its demand for sugar also increases. On the other hand, the arable land for sugarcane cultivation decreases. Therefore, raising cane productivity and sugar recovery is a never-ending effort to meet the increasing demand of sugar. One way to increase cane productivity is by improving genetic traits through breeding. However, genetic improvement of sugarcane has been hampered by its narrow genetic base. Modern commercial clones of sugarcane has been originated from only 20 noble sugarcane and fewer than 10 species of *Saccharum spontaneum* derivatives (Patade and Suprasanna 2008). Genetic diversity study using molecular marker also showed that modern commercial sugarcane clones have limited genetic base (Hapsoro et al. 2015; Devarumath et al. 2012; Tabasum et al. 2010; Khan et al. 2009) and it needs to be expanded.

One way of expanding genetic base is by induced mutation, which has been used to complement other breeding method to improve certain specific traits. Mutatation breeding is especially useful to modify one or few traits of good varieties without changing the rest of their characters. FAO and IAEA data of 2011 showed that mutation breeding has significantly contributed to crop improvement, as shown by the release of more than 3,079 improved mutant crop varieties for commercial cultivation (Shu et al. 2011). Mutation breeding can be conducted using chemical and physical mutagens such as irradiation. Use of physical mutagens has been more preferable than chemical ones since the former is more practical so that it is easier to employ. One of the physical mutagens commonly used in mutation breeding is gamma ray (Chopra 2005, Liu et al. 2004). Mutation breeding using gamma irradiation in commercial crops has been reported such as in soybean (Mudibu et al. 2012), peanut (Muthusamy et al. 2007; Lukanda et al. 2013), wheat (El-Sayed et al. 2007), tomato (Sikder et al. 2013), sweet potato (Shin et al. 2011) and sugarcane (Patade et al. 2008; Patade and Suprasanna 2009).

One way of mutation breeding in sugarcane was to expose *in vitro* growing calli to gamma ray, regenerate the calli into plants, and then evaluate the plants (Nikam et al. 2015). Therefore, it is necessary to establish an in vitro plant regenaration system in sugarcane and to determine LD_{50} of gamma ray for irradiating sugarcane calli. Four clones of sugarcane, i.e. Ragnar, X3, GM19, and GP11, found in the collections of Gunung Madu Plantations Co., Lampung, Indonesia were used as these displayed high sugar yield and have become important germplasm for sugarcane breeding program. In this paper, we report plant regeneration of sugarcane calli, and using the regenaration system, the radiosensitivity of the calli to various dosage of gamma ray was assessed to determine the LD_{50} of the gamma ray for mutation breeding in sugarcane.

MATERIALS AND METHODS

Callus Induction

Explants were leafrolls as previously described (Hapsoro et al. 2012). Approximately 15-cm young shoots, derived from sugarcane cv. Ragnar, X3, GM19 and GP11 were cut off and several sheaths were removed from nodes, resulting in shoot rods. For surface sterilization, the shoot rods were soaked for 30 seconds in ethanol 70% and shaken for 10 minutes in NaOCl (sodium hypochlorite) 1.3%, followed by three rinses with sterile distilled water. Leafrolls (4-5 mm thick), which was obtained by cutting the shoot rods, were cultured on callus-inducing medium (CI) to induce calli. The CI medium consisted of MS salts (Murashige and Skoog,1962), 30 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol, 150 m L^{-1} coconut water, 0.1 mg L^{-1} thiamine-HCl, 0.5 mg L^{-1} pyridoxine-HCl, 0.5 mg L^{-1} nicotinic acid, 2 mg L^{-1} glycine, and 3 mg L^{-1} 2,4-D. The pH of the medium was adjusted to 5.8 before being added with 8 g L^{-1} agar as a solidifying agent. The medium mix was autoclaved at 121 ^{o}C and 1.5 kg cm^{-2} for 20 minutes. Explants were cultured on CI medium, 4 explants per 250 ml culture bottle, and the cultures were incubated in dark culture room at 26 ± 2 ^{o}C. After 4 weeks, the proliferating calli were subcultured on the same medium. Callus clumps of 0.26-0.30 g were subcultured on the same medium for 4 weeks. Clones were used as treatments, 3 bottles per treatment, and 4 callus clumps per bottle. The experiment was repeated three times. At 8 weeks of culture, the proliferation of calli was estimated using scores described as follows. Score 0 = calli less than 0.25 g. Score 1 = 0.25-0.50 g. Score 2 = 0.50-0.75 g. Score 3 = 0.75-1.00 g. Score 4 = more than 1.00 g. Callus clumps of approximately 0.25 g and 1.00 g were weighed. The amount of calli from the two weighed value was used to determine the scores. Data were subjected to analysis of variance and the mean separation was conducted using least significant difference ($LSD_{0.05}$).

Shoot induction

The calli was subsequenly subcultured to shoot-inducing medium (SI) to induce shoots. The composition of SI medium was the same as CI medium except that the plant growth regulator was 2.5 mg L^{-1} of benzyladenine (BA) and no coconut water was included. Media preparation was conducted

as previously described. Calli of each clone was cultured in clumps arranged in circles having diameters of 0.5 cm, 1 clump per culture bottle (250 ml in volume), 8 culture bottle per treatment. Cultures were mantained in a culture room at 26 ± 2 ºC and provided with fluorescent lamps of approximately 1000 lux using 16-8 photoperiods. Subcultures to the same medium were conducted every 4 weeks and at 8 weeks in culture data on shoot formation were recorded using scores described as follows. Score 1= 1-10 fully developed shoots/callus clump. Score 2: 11-20 fully developed shoots/callus clump. Score 3 = 21-30 fully developed shoots/ callus clump. Score 4 = more than 30 fully developed shoots/callus clump. Data were subjected to analysis of variance and the mean separation was conducted using least significant difference ($LSD_{0.05}$). After 8 weeks in culture the growing shoot clusters on calli were subcultured on shoot-elongating (SE) medium, which was the same as the SI medium, except that it was added with 2 g L^{-1} activated charcoal and not supplemented with plant growth reglators. After 4 weeks in SE medium, the shoots were ready for rooting. All cultures were mantained in a culture room at 26 ± 2 ºC and provided with fluorescent lamps of approximately 1000 lux using 16-8 photoperiods.

Rooting and acclimatization

Clusters of shoot of cv Ragnar were cultured on root-inducing (RI) medium containing different concentrations of indolebutyric acid (IBA) (0, 2, 5, 7.5, and 10 mg L^{-1}). The RI medium was the same as the SI medium except for the plant gowth regulators. The medium was contained in a 350 mL-culture bottle and all cultures were put under fluorescent lamp of approximately 1000 lux at 26 ± 2 ºC and 16-8 photoperiods. Each treatment consisted of 9 culture bottles, each containing one cluster of 11-17 shoots. After 4 weeks in culture, shoots rooted, and the clusters of plantlet in culture bottles were hardened off for 1 week by placing them on a laboratory bench beside windows to expose them to diffused sunlight. Plantlet clusters were taken out from the bottles, and number of roots was recorded. Roots were washed under running tap water to get rid of agar. The washing was carefully done in order that the plantlet clusters were kept intact. Upper part of shoots was cut off, resulting in plantlet clusters having shoots of 10 cm in length. Plantlet clusters were soaked in 2 g L^{-1} fungicide solution for 10 minutes then rinsed with water.

Plantlet clusters (11-17 planlets per cluster) from different rooting treatments were acclimatized using media consisting of sand and compost (1:1 v/v) contained in pots (6.6 cm in diameter), one cluster per pot. Each treatment consisted of 9 pots. Each cluster was covered with transparent plastic bag to maintain humidity and placed in shaded glasshouse. One week later the cover was removed and the plants were grown for 8 weeks. Still in clusters, the plants were then transplanted to polybags containing a mixture of soil and compost (1:1 v/v) and placed under full sunshine. Both rooting and acclimatization experimens were arranged in a completely randomized design with three replications. Data were subjected to analysis of variance and the mean separation was conducted using least significant difference ($LSD_{0.05}$).

Response of callus to gamma irradiation

Calli of sugarcane cv. Ragnar were generated as previously described. Clumps of calli were selected and cultured for 2 weeks on CI medium contained in a Petri dish in a dark culture room, and subjected to accute gamma irradiation (0, 5, 10, 15, 20, 25, 30, 40, 50, 60 Gy) which was conducted at National Nuclear Energy Agency of Indonesia (BATAN), Jakarta, Indonesia. Each treatment consisted of 12 Petri dishes, each containing 10 callus clumps. The callus clumps was irradiated and one day after irradiation the cultures were put on fresh CI medium and incubated in the dark culture room for 4 weeks. Percentage of surviving callus clumps which contained embryogenic calli was calculated. Embryogenic calli have the characteristics of being compact, white in color, and not translucent. The experiment was arranged in a completely ramdomized design with three replicates and the data were subjected to regression analysis by plotting the dosage of gamma irradiation against the percentage of surviving callus clumps. Based on this regression analysis, the LD_{50} of gamma irradiation was determined.

RESULTS AND DISCUSSION

Plant regeneration

In vitro plant regeneration of sugarcane in this study consisted of four steps, i.e callus induction, shoot induction, root induction, and plantlet acclimatization (Fig. 1). Leafrolls of four different clones were cultured on callus-inducing media and their response was studied. Calli started to appear at second week of culture and then proliferated. Analysis of variance showed that callus induction was significantly affected by clones. Table 1 showed that clone X3 was found to be the most responsive in term of callus formation among the clones studied, followed by GM19, GP11, and Ragnar, suggesting that genetic factors influenced plant regeneration *in vitro.* On the other hand, analysis of variance showed that shoot formation was not significantly affected by clones (Table 1). This might be caused by the use of selected calli for shoot formation in this experiment so that they might have the same morphogenetic capacity. The selected calli for shoot formation were those having the characteristics of being compact, hard, smooth-looking, and not translucent. Such characteristics of callus indicate their embryogenicity in sugarcane tissue culture (Lakshmanan 2006; Ho and Vasil 1983).

Table 1. Response of leafroll explants of different sugarcane clones to callus-inducing medium and shoot-inducing medium *in vitro.*

No.	Clones	Callus scores *	Shoot scores**
1	Ragnar	1.4 b	1.5
2	X3	2.4 a	2.0
3	GM19	2.1 a	1.5
4	GP11	1.9 ab	1.5

*Means followed by same letters do not differ according to LSD 0.05.
** non-sgnificance according to analysis of variance

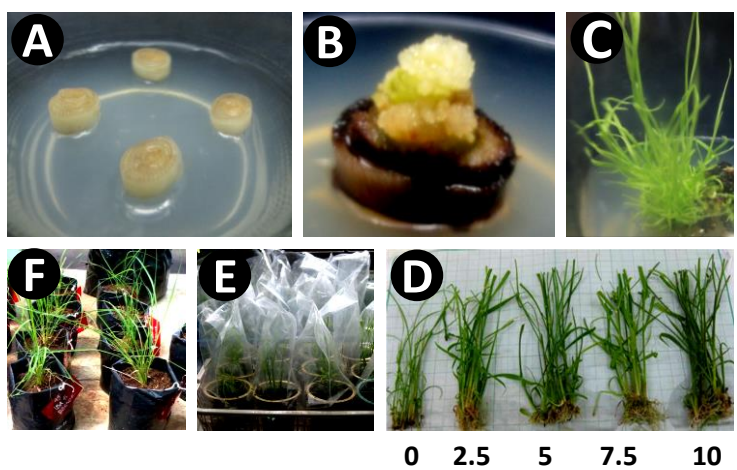

Fig. 1. Plant regeneration of sugarcane calli cv. Ragnar. Leafrolls were cultured on callus-inducing medium to produce calli (A). The calli (B) was then transferred to shoot-inducing medium to produce shoots (C). Clusters of shoots were rooted on root-inducing media containing IBA (D). Numbers indicate IBA concentrations (mg L^{-1}). The plantlets were then acclimatized (E) and then grown in a greenhouse (F).

Elongated shoots were then used for rooting experiment. Only clone Ragnar was used in this experiment since this clone has become one of the important sugarcane germplasms for breeding

program in Gunung Madu Plantations Co. Clone Ragnar was selected as superior variety among 26 clones based on maturity earliness, juice parameters, ton sugar per hectare, and balanced fibre content (Wagih et al. 2004). Clone Ragnar was also considered as drought tolerant (Wagih et al. 2001).

The addition of IBA on RI medium resulted in increased number of roots (Fig. 2, Figure 1D). Further increase in IBA concentration up to 5 mg L^{-1} led to increased number of roots, but higher concentration up to 10 mg L^{-1} resulted in decreased number of roots (Fig. 2).

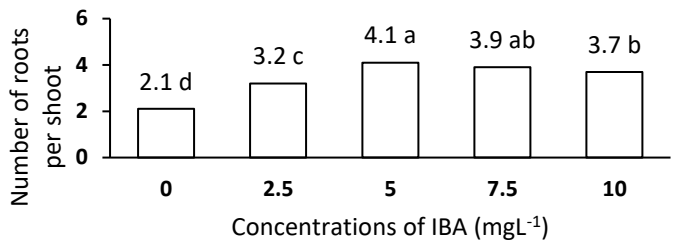

Fig. 2. Effects of IBA concentrations on number of roots. Means followed by same letters do not differ according to LSD0.05.

Some researchers also used IBA for root induction in sugarcane tissue culture (Yasmin et al. 2011; Mustafa and Khan 2016; Gopitha et al. 2010, Abu et al. 2014; Varakagoda et al. 2007) while other researchers used NAA (Nikam et al. 2015; Dinesh et al. 2015; Getnet et al. 2016) and without plant growth regulators (Munsamy et al. 2013; Koch et al. 2012) for optimum root induction.

Rooted shoots were then acclimatized to external conditions. The plantlets derived from IBA-treated shoots showed better survival rate than those from the untreated ones (Fig. 3). An increase in IBA concentrations up to 5 mg L^{-1} resulted in increased survival rate of plantlets; further increase up to 10 mg L^{-1} led to decreased survival rate. The highest survival rate (68.4%) was achieved when shoots were previously treated with 5 mg L^{-1} IBA (Fig. 3). These results might be related to the number of roots being produced per shoot. The highest number of roots was achieved on media supplemented with 5 mg L^{-1} IBA. More roots might support more favourable nutrient uptake, leading to higher survival rate.

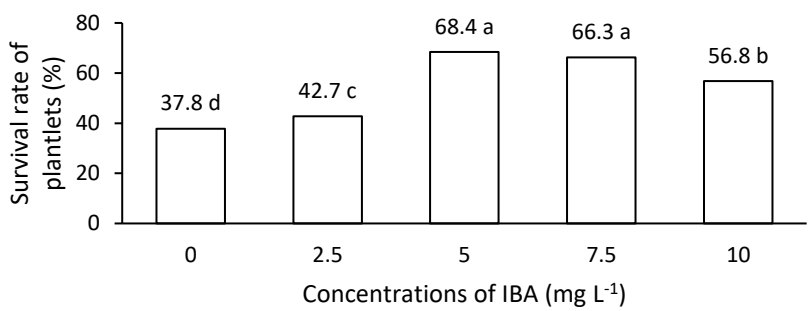

Fig. 3. Survival rate of sugarcane plantlets undergoing acclimatization. Plantlets were derived from shoots treated with different concentrations of IBA. Means followed by same letters do not differ according to LSD0.05.

Response of callus to gamma irradiation

The survival rate of calli decreased with increasing dosage of gamma ray from 0 to 40 Gy, and no clumps produced embryogenic calli when the dosage reached 50 and 60 Gy (Fig.4.and Fig.5.). A

regression analysis showed that the value of LD_{50} was 17 Gy, lower than that reported by other researchers. Using relative growth rate, Nikam et al. (2015) concluded that LD_{50} for radiosensitivity of sugarcane calli to gamma ray irradiation was 20 Gy. Patade and Suprasanna (2009) also used gamma irradiation of 20 Gy as LD50 to induce mutation in sugarcane calli for salinity tolerance. The value of 20 Gy as LD_{50} for gamma irradiation of sugarcane calli was also reported by other researchers (Saif-Ur-Rasheedet al. 2001; Patade et al. 2008).

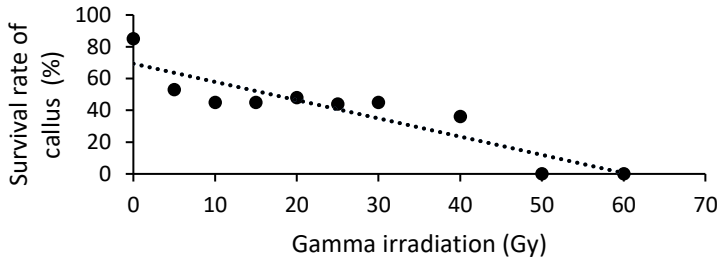

Fig. 4. Effect of gamma irradiation on survival rate of sugarcane callus *in vitro*. The survival rate was stated in percentage of callus clumps with proliferating embryogenic calli.

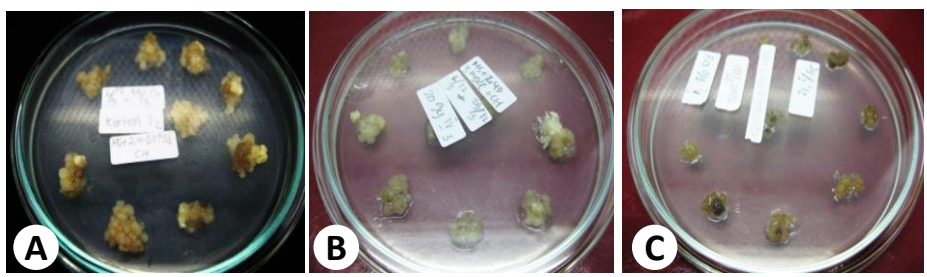

Fig. 5. Response of sugarcane callus to gamma irradiation. Data were recorded after irradiated clumps of callus were cultured for 4 weeks on fresh callus-inducing medium. (A) Without irradiation. (B) After being irradiated with gamma ray 20 Gy and (C) 60 Gy.

The damage of living cells caused by exposure to gamma irradiation is mainly through the indirect effects of the ray (Han and Yu 2009). Since living cells mostly consists of water, as they are exposed to gamma irradiation the water molecule releases free radicals which are destructive to the cells (Han and Yu 2009). Since our experiment used callus clumps placed on semi solid media containing water when irradiation was conducted, the water in this media might also release the free radicals. That might be the reason for the lower LD_{50} obtained in this experiment (17 Gy) compared to other reports, where the LD_{50} was 20 Gy, in which the calli were taken from the media and subjected to gamma irradiation (Saif-Ur-Rasheedet al. 2001; Patade et al. 2008; Patade and Suprasanna 2009; Nikam et al.2015).

In vitro plant regeneration system, plantlet acclimatization, LD_{50} of gamma irradiation from this study could be applied to *in vitro* based mutation breeding of sugarcane. Basically, this could be done by irradiating the proliferating sugarcane calli with LD_{50} of gamma ray, regenerating the irradiated calli into plants, and selecting the plants with desired characters. *In vitro* based mutation breeding in sugarcane by irradiating proliferating calli has been reported for other sugarcane cultivars (Zambrano et al. 2003; Ali et al. 2007; Patade et al. 2008; Kaur and Gosal 2009; Patade and Suprasanna 2009; Yasmin et al. 2011; Maria et al. 2012; Nikam et al. 2015).

CONCLUSIONS

In vitro shoot regeneration system using leafrolls as explants was established for sugarcane cv. Ragnar, X3, GM19 and GM11. A plant regeneration system was also established for clone Ragnar. Calli were induced and proliferated on CI medium containing 3 mgL^{-1} 2,4-D, subcultured onto SI medium containing 2.5 mgL^{-1} BA for shoot formation, and subcultured onto SE medium devoid of plant growth regulators for shoot elongation. Shoots were rooted on RI medium containing 5 mg L^{-1} of IBA and the rooted shoots (plantlets) were acclimatized using standard procedures. This regeneration system could be used in mutation breeding in sugarcane by exposing the proliferating calli to gamma irradiation. Using our system, a dosage of 17 Gy was found to be the LD_{50} of gamma ray for irradiating calli of sugarcane cv. Ragnar. This is the first report for the gamma irradiation of calli of sugarcane cv. Ragnar.

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