International Journal of Sciences: Basic and Applied Research (IJSBAR)

ISSN 2307-4531
(Print & Online)

http://gssrr.org/index.php?journal=JournalOfBasicAndApplied

Gamma Irradiation and *In vitro* Selection Could Increase Drought Tolerance in Sugarcane

Sri Suhesti[a]*, Nurul Khumaida[b], Gustaaf Adolf Wattimena[c], Muhamad Syukur[d], Ali Husni[e], Endang Hadipoentyanti[f]

[a,e,f]*Indonesian Agency for Agricultural Research and Development (IAARD), Jl Ragunan 29 Pasar Minggu Jakarta Selatan 12540, Indonesia*

[b,c,d] *Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University, Darmaga Campus, Bogor 16680, Indonesia*

[a]*Email: hesti.khrisnawijaya@gmail.com*

[b]*Email: nkhumaida@yahoo.com*

Abstract

Drought is one of the problems that decrease sugarcane productivity. Therefore it needs to develop a new drought tolerant sugarcane variety. The objectives of this research were to evaluate the response of gamma irradiated calli and drought tolerant of putative mutants through *in vitro* and *in vivo* selection. Kidang Kencana (KK) variety was used as mother plant in this study. It has high productivity but susceptible to drought stress. Embryogenic calli were induced on MS media supplemented with 9 µM 2.4-D + 4.5 µM Picloram. Six levels of Gamma rays irradiation were used (0, 10, 20, 30, 40 and 50 Gy), while two levels of Polyethylene Glycol (PEG) were used for *in vitro* selection (0 and 10%). The plantlets derived from *in vitro* selection were acclimatized and selected in greenhouse. These putative mutants were treated with drought and watering condition (control). The result showed that irradiated calli at 10 and 20 Gy were more tolerant to 10% PEG selection media compared to the negative control (0 Gy). Among 42 obtained, the 17 putative mutants had higher drought tolerance than their negative and positive controls.

---

* Corresponding author.

E-mail address: hesti.khrisnawijaya@gmail.com

However, only four putative mutants had higher drought tolerance level than positive control (PSJT 941). It was concluded that gamma irradiation continued with *in vitro* and *in vivo* selection could increase drought tolerance of sugarcane.

Keywords: *Saccharum officinarum* L.; Induced mutation; polyethylene glycol; somaclone; selection.

1. Introduction

Sugarcane (*Saccharum officinarum* L.) is one of the industrial plants which produces 65% sugar. Drought is one of the factors that decreased its productivity. Quantitatively, yield losses due to drought achieve to 40% of the potential production if occur in the critical phase of the plant [1]

The effort to improve sugarcane productivity can be performed by extensification. Because of the limits productive land, it should be planted at marginal land, It has some limiting factors for plant growth, such as drought.

Sugarcane drought-tolerant varieties can be performed by single or combined methods of conventional, and mutation breeding or *in vitro* selection and genetic engineering. The conventional breeding through hybridization has been used to overcome this problem. However there are several constraints, included complex genome, low fertility [2] and high ploidy level [3].

The combined method of mutation and *in vitro* selection is a potential tool to be applied. The drought-tolerant mutants can be obtained by applying *in vitro* selection and selective agents of polyethylene glycol (PEG). This compound can be used for simulating drought conditions at the field. There were several reports of increasing drought tolerance gamma ray irradiation and *in vitro* selection on sorghum [4], sugarcane [5] and wheat [6]. The objectives of this research were to evaluate the response of gamma irradiated calli and drought tolerant of putative mutants through *in vitro* and *in vivo* selection.

2. Materials and Methods

Kidang Kencana variety used in this research. This variety is high productivity but drought sensitive.

2.1. Embryogenic callus induction

Callus was inducing from roll leaf explants. Surface sterilization is conducted by 96% alcohol and passed over Bunsen flame in the laminar air flow cabinet. The sterile explants were cut into ± 0.5 cm size and planted on MS callus induction media with the addition of 9 μM Dichlorophenoxy acetic acid (2.4 –D) + 4.5 μM Picloram and 1 g/l casein hydrolisate, 3% sugar, and 0.25% Phytagel. Media were adjusted at 5.8% pH [7]. The cultures were incubated at 20-22^{o}C with dark condition for 12 weeks. Subcultures were conducted every four weeks on the same medium for twice.

2.2. Gamma irradiation of embriogenic calli

Embryogenic calli obtain from the previous step were irradiated by gamma ray (Gamma Chamber 4000A with active compound of Co^{60} This research was arranged in completely randomized design with 6 doses 0, 10, 20, 30, 40 and 50 Gy [8]. Each treatment consisted of 10 replications. Each replication consists of 5 clumps of calli. Irradiated calli were sub cultured on hormone free media (MS0) for 2 weeks. Subsequently, those calli were regenerated on regeneration 2.46 µM IBA + 1.33 µM BAP [7] for two months. They were incubated in culture room with ± 1000 lux light intensity for 16 hours periodicity. The observed variable was percentage of survival calli. The data was analyzed by Curve Expert Program 1.3 to determine LD_{20} and LD_{50}.

2.3. In vitro selection of gamma irradiated mutants for drought tolerance

The two weeks gamma irradiated embryogenic calli that planted on MS0 media were transferred to liquid selection media (MS0 + PEG 6000). Selection was conducted for 12 weeks. The survived calli were sub cultured on regeneration media. They were incubated in culture room with ± 1000 lux light intensity for 16 hours periodicity at temperature of 20-22^{0}C. The survival plantlets (M_1V_0) were regenerated to form M_1V_1 - M_1V_3 population. The experiment was arranged in factorial completely randomized design with 10 replications. The first factor was the concentration of PEG 6000 (0 and 10%) [9]. The second factor was the dose of gamma ray irradiation (0, 10, 20, 30, 40, 50 Gy). Each replication consisted of 5 clumps calli. The observed variables were percentage of survival callus, number and height of shoots.

2.4. In vivo selection of putative mutants for drought tolerance

The vigorous plantlets were acclimatized on soil: manure (2:1) for 1.5 months in greenhouse. The survival seedling were transferred to seven kg media of soil: sand: manure (2:1:1) for 3.5 months. The 42 putative mutants (five months after acclimatized in green house), the mother plant of Kidang Kencana as negative control and drought tolerant variety of PSJT 941 as positive control. Fertilizers were added to the media according to the standard operating procedures [10]. The putative mutants were not watered for 23 days to give drought stress, when more than 90% of the negative control plants had the folding and wilting leaves (L+1, the first leaves that have ear leaf). Control treatment was done with regular watering until field capacity. This research was arranged in factorial completely randomized block design with two replications. The first factor was putative mutants (42 numbers) and the second factor was drought treatment (drought and watering). The observed variable was percentage of folding and wilting leaves of the D+1 leaf.

2.5. Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) with confidence level of 95%. If there was a significant difference among treatments, further analysis performed using Duncan test (Duncan Multiple Range Test/DMRT) with confidence level of 95%. Analysis Dunnett test was done to determine the mutants which have higher drought tolerance compare the negative and positive control.

3. Results

3.1. Embryogenic callus induction

The initial response of callus formation was leaves swelling accompanied by the emergence of callus, especially around the wounding. Embryogenic callus was produce on callus induction. The visual performnace of the calli were dry, friable and white milk or cream [7]. According to [11], callus with these characteristics is embryogenic callus. This embryogenic callus was successfully initiated for 14 days. The percentage of explants forming callus was 98% and the percentage of embryogenic callus was 94% [7].

3.2. Gamma irradiation of embryogenic calli

After treated with gamma irradiation, embriogenic calli turn to brown and black colour, especially occurred in high doses irradiation. Statistical analysis of survived calli decreased with the increasing of irradiation doses. The highest percentage of survival calli found in the control treatment (0 Gy) and was not significantly different with irradiation treatment of 10 and 20 Gy. The lowest percentage of survived calli yielded from 50 Gy, but not significantly different with 40 and 30 Gy (Table 1). This result was similar with the previous research [5].

Table 1: The survived calli of sugarcane variety Kidang Kencana on several doses of gamma ray irradiation

Irradiation Doses (Gy)	Survived calli (%)
0	62.50 a
10	52.40 a
20	53.00 a
30	16.90 bc
40	22.20 b
50	7.30 c

Note: Number followed by the same letters in the same column showed no significant difference in the level of 5% DMRT.

Result of the lethal dose calculation using curve fit analysis program on variable of percentage of survived callus indicates that the best model equation Y = 103.14 - 1.84 x. Thus, it found that the lethal dose of LD_{20} was 12.57 Gy and LD_{50} was 28.88 Gy. These results are same with the research of [8] and [12] which yielded that the lethal dose 50 (LD_{50}) of sugarcane calli with gamma irradiation treatment was at dose of 20 Gy. The calli resulted from the treatment between LD_{20} and LD_{50} doses expected to have high variability and provide a higher probability to get certain properties for purpose breeding.

3.3. In vitro selection of gamma irradiated mutants for drought tolerance

There was significant interaction between the dose irradiation and selection media to the survived calli variable. The percentage of survived callus on control media (0% PEG) decreased with the increase of irradiation doses. The dose of 10-30 Gy was significantly increasing the survival rate of calli, indicated by the number of relative decrease index (Table 2). It means that those doses could increase the tolerance to calli to 10% PEG.

The survived calli was lower in 10% PEG selecting media for 60 days than the control (0% PEG). It means that

10% PEG can be used as selecting agent for drought tolerance. The same statement was also reported by the other researcher [9] when the PEG was treated for 40 days. The PEG treated calli become reddish and several of them turn to black

Table 2: The effect of combined treatment of gamma irradiation and *in vitro* selection with PEG to the survive calli of sugarcane variety Kidang Kencana, 60 days

Irradiation Dose	**Percentage of callus survival**		**Relative decrease**
(Gy)	0% PEG	10% PEG	**index (%)***
0	100.00	66.00	34.00
10	97.00	81.50	15.98
20	91.30	71.00	22,23
30	74.00	69.20	3.89
40	72.00	31.00	56.94
50	66.50	19.70	70.38

Note:

- There was significant different between gamma irradiation and PEG treatment.
- *) The relative decrease index was calculated by dividing the number of survived calli from the 10% PEG with the 0% PEG multiplied by 100.

There was no interaction between irradiation treatment and selecting media to shoots number and height, but these single treatments were significantly affected to those variables (Figure 1). The higher shoot number and height resulted from the 10 and 20 Gy dose than the control. Those doses were around LD 20 and LD50. It suggested that those doses may increase growth rate. The 10% PEG selection media caused the decrease of shoots number and height.

The visual performance of putative mutants was shown at Figure 2. The shoot growth of 10 to 20 Gy irradiated cultures (Figure 2b, 2c, 2h, 2i) was better than the other treatments both on selecting media and the control and PEG treatment. Among the plantlets, about 80% was successfully acclimatized and totally obtained 42 putative mutants.

3.4. In vivo selection of putative mutants for drought tolerance

There were different responses among 42 putative mutants to the growth under drought stress tested in greenhouse. Several mutants showed different response to drought stress compared to the negative and positive control, Kidang Kencana and PSJT 941 respectively. The early drought symptoms of sugarcane could be detected from the rolling leaves at the tip area during the day, but it could recover in the evenings. At more severe levels of drought stress, the leaf blades were rolled permanently and they could not recover and followed by wilting. This process was started from the older to the younger leaves. The wilting process began from the tips, to the edges of leaves and move to the middle leave and midrib. Finally the wilting process was occurred in

the sheaths. The rolled leaf as caused by the degrees of cell turgor. The damage can be observed after five days in drought stress.

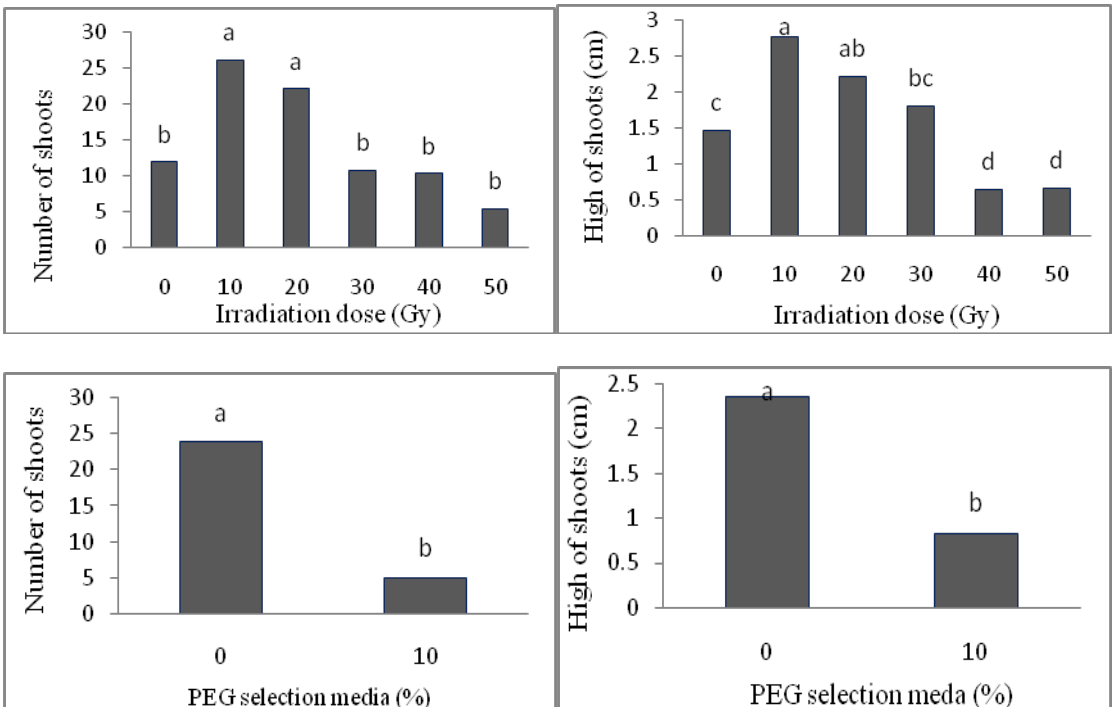

Figure 1: The effect of single treatment of irradiation dose and PEG selection media to the shoots number and height of sugarcane variety Kidang Kencana

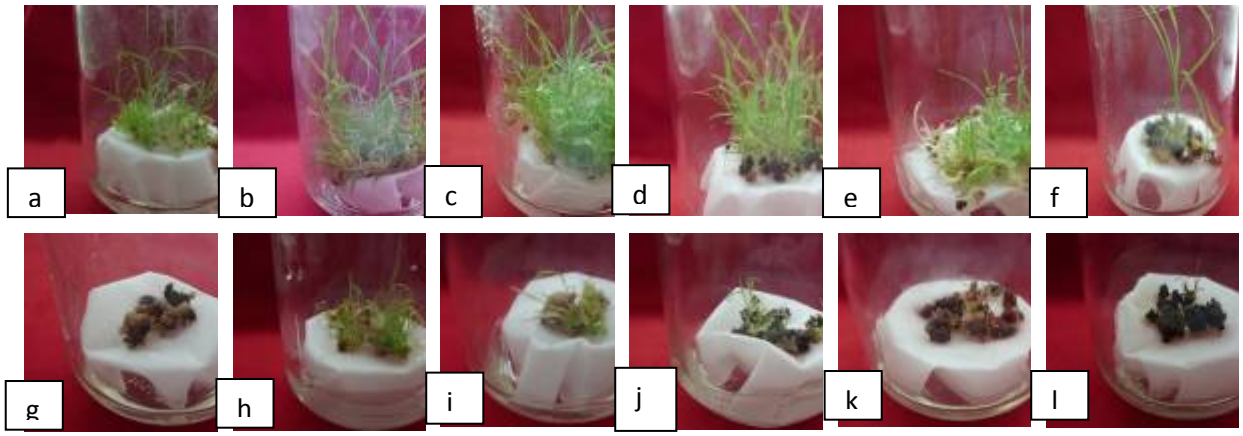

Figure 2: Visual performance of regenerated calli of sugarcane from variety Kidang Kencana after gamma irradiation (upper row) and *in vitro* selection treatment with 10% PEG (lower row), 2 months after treatments: control 0 Gy (a and g); 10 Gy (b and h); 20 Gy (c and i); 30 Gy (d and j); 40 Gy (e and k); 50 Gy (f and l).

Table 5: Leaf damage of D + 1 leaves on 42 putative mutants variety Kidang Kencana and their control, 23 days after drought stress

No	Genotype	Leaf Damage (%)		Mutant test to the control				No	Genotype	Leaf Damage (%)		Mutant test to the control			
				KK(-)		PSJT 941(+)						KK (-)		PSJT 941 (+)	
		G	K	G	K	G	K			G	K	G	K	G	K
1	MK22 (0 Gy)	86.5	70.0		*			23	MK21 (20 Gy)	62.5	42.5		*		*
2	MK23 (0 Gy)	72.5	57.5		*			24	MK33 (20 Gy)	87.5	75.0				
3	MK24 (0 Gy)	67.5	57.5		*			25	MK34 (20 Gy)	60.0	45.0		*		*
4	MK25 (0 Gy)	60.0	37.5		*		*	26	MK35 (20 Gy)	72.5	82.5				
5	MK26 (0 Gy)	45.0	27.5	*	*		*	27	MK36 (20 Gy)	97.5	72.5				
6	MK27 (0 Gy)	37.5	27.5	*	*	*	*	28	MK37 (20 Gy)	50.0	42.5	*	*		*
7	MK28 (0 Gy)	55.0	30.0	*	*		*	29	MK38 (20 Gy)	85.0	62.5		*		
8	MK29 (10 Gy)	65.0	70.0		*			30	MK39 (20 Gy)	42.5	84.0	*		*	
9	MK96 (10 Gy)	67.5	37.5		*		*	31	MK40 (20 Gy)	55.0	60.0	*	*		
10	MK10 (10 Gy)	55.0	50.0	*	*		*	32	MK46 (20 Gy)	27.5	25.0	*	*	*	*
11	MK12 (10 Gy)	55.0	37.5	*	*		*	33	MK47 (20 Gy)	42.5	37.5	*	*	*	*
12	MK13 (10 Gy)	67.5	42.5		*		*	34	MK54 (20 Gy)	70.0	65.0		*		
13	MK14 (10 Gy)	75.0	50.0		*		*	35	MK57 (20 Gy)	45.0	32.5	*	*		*
14	MK15 (10 Gy)	47.5	38.5	*	*		*	36	MK58 (20 Gy)	45.0	47.5	*	*		*
15	MK16 (10 Gy)	50.0	47.5	*	*		*	37	MK51 (30 Gy)	50.0	42.5	*	*		*
16	MK17 (20 Gy)	100.0	80.0					38	MK42 (30 Gy)	70.0	72.5				
17	MK18 (20 Gy)	65.0	47.5		*		*	39	MK60 (40 Gy)	55.0	37.5	*	*		*
18	MK30 (20 Gy)	100.0	92.5					40	MK64 (40 Gy)	62.5	60.0		*		
19	MK31 (20 Gy)	70.0	37.5		*		*	41	MK65 (40 Gy)	100.0	100.0				
20	MK32 (20 Gy)	75.0	67.5		*			42	MK68 (40 Gy)	97.5	90.0				
21	MK19 (20 Gy)	39.5	37.5	*	*	*	*	43	KK (-)	100.0	100.0				
22	MK20 (20 Gy)	57.5	32.0	*	*		*	44	PSJT 941 (+)	85.0	85.0				

Note: G = roll leaf, K = wilting leaf, Kidang Kencana (KK) as a negative control, PSJT 941 as a positive control. Mutants are marked with asteric sign (*) indicate significantly different from the negative control or positive control based on Dunnett test at level of 5%

Base on table 5, there were 17 putative mutants that have better drought tolerance level than negative control (KK), but only four putative mutants that have better drought tolerance than positive control (PSJT 941). It was determined by the value of leaf damage where the leaf damage (rolling and wilting D+1 leaf) was significantly lower than the controls.

Among the 17 putative mutants which have increase drought tolerance, three of them derived from non

irradiated calli (MK 26, 27 and 28) and 14 mutant derived from irradiated calli consisted of four mutants from 10 Gy (MK 10, 12, 15 and 16), eight mutants from 20 Gy (MK 19, 20 , 37, 40, 46, 47, 57.58), one mutant from 30 Gy (MK51) and one mutant from 40 Gy dose (MK 60). The four putative mutants with higher drought tolerance than the positive control (PSJT 941), namely MK 27 (derived from non irradiated calli) and three mutants derived from 20 Gy irradiated calli (MK 19, 46, 47). It is suggested that somaclonal variation plays important role to form the three non irradiated mutants increase genetic variability in three mutants.

Drought tolerant mutants derived from the 20 Gy irradiation treatment, which around the LD_{50}. It indicates that the induced mutation by gamma irradiation especially at 20 Gy, provided greater opportunities to obtain specific mutants with desired properties.

4. Discussion

Gamma ray irradiation is widely used to improve plant genetic resources because it is very efficient to change the genetic materials. This physical mutagen has high energy and penetration power. When plant material is exposed to this mutagen, ionization process will occur in the tissues and causes changes at the cellular level, genomes, chromosomes and DNA [13].

Gamma irradiation affects plant responses depended on the type of culture and the rate of irradiation. In this research, calli were exposed by gamma irradiation. Calli are non differentiated cells, thus the probability of genetic chances is higher than differentiated cell (organ). This statement is similar with [14]. In this studi, the black and the brown irradiated calli is occurred because of the activity the polyphenol oxidase enzyme. According to [15] , the oxidation of phenolic compounds was occurred after the degradation of cell membrane or cell disorganization followed by degradation of chlorophyll. The brown color indicate the form of quinone formation [15]

Generally, the low dose gamma irradiation is applied to the *in vitro* culture [16, 17]. Sensitivity to irradiation can be measured based on the value of LD (lethal dose), which cause the cell death from the irradiated cells. The level of sensitivity is influenced by plant species, genotypes, growth stages, size, and explants type [18]. Some studies indicate that the optimum dose to produce mutants commonly obtained from the around of LD dose [19]. The highest variability was derived from the LD 20 and LD 50, in this research ranged between 10-30 Gy. [14] confirmed that 10-30 Gy gamma irradiations could increase somaclonal variation of non differentiated cells. [8] and [12] also found the lethal dose of LD50 occured at 20 Gy of sugarcane. Percentage of survival calli decreased with the increase irradiation dose. This is consistent with research of [5].

In vitro selection using drought selective agent of PEG was performed to obtain drought-tolerant sugarcane genotypes. This research showed that the use of 10% PEG for two months capable to inhibit the survival and regeneration rate of calli, which indicated by relative decrease index of the control was 34%. This is consistent with [9] which stated that callus proliferation and regenerated sugarcane plantlets would be restricted by *in vitro* selection using selecting agent 10% PEG.

It was observed that the 10 and 20 Gy irradiated calli increased their ability for surviving and regenerating become plantlets compared to the control on selecting agent of 10% PEG. This corresponds [16] and [20] which states that the use of low dose irradiation can stimulate *in vivo* plant growth.

The use of PEG during *in vitro* selection can induce of water stress and has positive correlation with drought stress at field and greenhouse [21]. This results give strong expectation that the survived calli during *in vitro* selection would have better drought tolerance when tested in greenhouse or field.

The starting materials in this research were rolled leaf explants and the regeneration system was somatic embryogenesis which through the callus formation. That is why the non irradiated calli could generate drought tolerant putative mutants at low frequency. It is probably due to polysomic tissue on sugarcane leaf explants and non differentiated callus formation that cause somaclonal variation. Somaclonal variation is spontaneously genetic variation as a result of tissue culture process using somatic cells.

On the early growing stage, sugarcane needs successive water. It shows morphological and physiological disorders if water is limit. Effect of drought stress in one variety is different with the other varieties [22]. There are 17 putative mutants obtained in this research during *in vivo* at green house. These mutants had higher drought tolerance level than the negative control. It means gamma irradiation followed by *in vitro and in vivo* selection may increase drought tolerance in sugarcane. Evenly, the four putative mutants had higher drought tolerance than the positive control (PSJT 941). It means the induce mutation by gamma irradiation and *in vitro* selection by 10% PEG provided superior mutants.

4. Conclusion

Percentage of survived calli decreased with the increase of gamma irradiation dose. The lethal doses of LD_{20} and LD_{50} were 12.57 Gy and 28.88 Gy, respectively. The 10 and 20 Gy increased drought tolerance level of sugarcane calli variety Kidang Kencana during *in vitro* selection. There were 17 putative mutants with higher drought tolerance than their negative and positive controls. However, only four putative mutants have drought tolerance higher than positive control (PSJT 941).

Acknowledgement

The authors thank Indonesian Center of Estate Crops Research and Development, Indonesian Agency for Agricultural Research and Development for giving the this research fund. Acknowledgement also gives to Dr. Ika Roostika Tambunan for valuable discussion during prepared this article.

Refferences

[1] G. Irianto. “Tebu Lahan Kering dan Kemandirian Gula Nasional.” Internet: www.litbng.go.id/artikel/one/28/pdf. April 24, 2003. [May 21, 2012].

[2] Suprasanna, V.A. Bapat. “Advances in the development of *in vitro* culture systems and transgenic in

sugarcane." *Proc. International Symposium on Technologies to Improve Sugar Productivity in developing countries, Guilin, China*. p. 629–636. 2006.

[3] R.A. Gilbert, M. Gallo Meagher, J.C. Comstock, J.D. Miller, M. Jain, Abouzid. Agronomic evaluation of sugarcane lines transformation for resistance to sugarcane mosaic virus strain E. *Crop sci*.: 45: 2060-2067. 2005.

[4] R.R. Duncan, R.M. Waskom, M.W. Nabors. "*In vitro* screening and field evaluation of tissue-culture-regenerated sorghum *(Sorghum bicolor* L) for soil stress tolerance." *Euphytica* 85: 371-380. 1995.

[5] V.Y. Patade, P. Suprasanna, V.A. Bapat, U.K. Kulkarni. "Selection for abiotic (salinity and drought) stress tolerance and molecular characterization of tolerant lines in sugarcane." Barc Newsletter Issue no 273 October 2006: 224-253. 2006.

[6] I. Mahmood, A. Razzaq, M. Ashraf, I.A. Hafiz, S. Kaleem, A. Qayyum, M. Ahmad. "*In vitro* selection of tissue culture induced somaclonal variants of wheat for drought tolerance." *J. Agric. Res.* 50(2): 177-188. 2012.

[7] S. Suhesti, N. Khumaida, G.A. Wattimena, M. Syukur, A. Husni, E. Endang, R.R. S. Hartati. Callus Induction and Plant Regeneration of Two Sugarcane Varieties (Saccharum officinarum L.) through *In vitro*. J. Littri. 21(2). 2015. [Indonesian] [In Press].

[8] V.Y. Patade, P. Suprasanna, V.A. Bapat. "Gamma irradiation of embryogenic callus cultures and *in vitro* selection for salt tolerance in Sugarcane (*Saccharum officinarum* L.)." *China Agr. Sci.* 7(9): 1147 – 1152. 2008.

[9] M.K. Begum, M.O. Islam, M.A.S. Miah, M.A. Hossain, N. Islam. "Production of somaclone *in vitro* for drought stress tolerant plantlet selection in sugarcane (*Saccharum officinarum* L.)." *The Agriculturists* 9 (1&2): 18-28 (Abstr.) 2011.

[10] Puslitbangbun. "Budidaya dan Pasca Panen Tebu. Pusat Penelitian dan Perkembangan Perkebunan." Badan Penelitian dan Pengembangan Pertanian. Jakarta (ID). Kementrian Pertanian. 2012.

[11] C.H. Gandonou, T. Errabii, J. Abrinii, M. Idaomari, F. Chibi, N.S. Senhaji. "Effect of genotype on callus induction and plant regeneration from leaf explants of sugarcane *(Saccharum* sp.)." *African J. Biotechnol.* 4(11): 1250-1255. 2005.

[12] M. Saif-Ur-Rasheed, S. Assad, Y. Zafar, R.A. Washeed. "Use of radiation and *in vitro* techniques for development of salt tolerant mutants in sugarcane and potato." *IAEA Tech. Doc.*1227: 51-60. 2001.

[13] F.I.S. Medina, E. Amano, S. Tano. Mutation Breeding Manual. Japan. Forum for Nuclear Cooperation in Asia (FNCA). 2005.

[14] A.M. Van Harten. "Mutation breeding Theory and Practical Aplication New York."*Cambridge University Press*. P. 342.1988.

[15] H. Laukkanen, L. Rautiainen, E. Taulavuori , A. Hohtola. “Changes in cellular structures and enzymatic activities during browning of scots pine calls derived from mature buds.” *Tree Physiol.* 20: 467-475. 2000.

[16] B.Al-Safadi , Z. Ayyoubi, A. Jawdat. “The effect of gamma irradiation on potato microtuber production *in vitro*.”*Plant Cell Tiss.* Org. Cult. 61:181-187.2000.

[17] G. La Vina, A.B. Munoz, F.P. Alfaro. “Effect of culture media and irradiance level on growth and morfology of *Persea americana* Mill microcutting.”*Plant Cell Tiss*. Org. Cult. 65:229-237.2001.

[18] B.K. Banerji, S.K. Datta. Gamma ray induced flower shape mutation in chrysanthemum cv Java. J. *Nuclear Agric*. Biol. 21(2):73-79. 1992.

[19] S.K. Datta. Recent Developments in Transgenic for biotic Tolerance in Rice. *JIRCAS Working Report* 43-53. 2002.

[20] A. Borzouei, M. Kafi, H. Khazaei, B. Naseriyan, A. Majdabadi. “Effects of gamma radiation on germination and physiological aspects of wheat (*Triticum aestivum* L) seeding.” Pak. J. Bot. 42:2281-2290.2010.

[21] K.C. Short, I. Warburton and A.V. Roberts. *In vitro* Hardening of Cultured cauliflower and *Chrysantenum* Plantlets to Humidity. *Acta Hort. 212:* 329-334. 1987.

[22] M.A. Silva, J.A. G da Silva, J. Enciso, V. Sharma, J. Jifon. Yield component as indicators drought torerance of sugarcane. *Sci. Agric*. 65(6) : 620-627. 2008.