In vitro propagation of six selected sugarcane mutant clones through leaf explants

R Purnamaningsih¹,*, D Sukmadjaja¹, S Subesti², and S Rahayu¹

¹ Balai Besar Penelitian dan Pengembangan Bioteknologi dan Sumberdaya Genetik Pertanian, Jl. Tentara pelajar no.3A Bogor, Indonesia
² Pusat Penelitian dan Pengembangan Perkebunan, Jl. Tentara Pelajar No. 1 Bogor, Indonesia

* E-mail: raga_padmi@yahoo.com

Abstract. Six mutant clones of sugarcane with high productivity have been produced through tissue culture techniques combined with mutations using gamma-ray irradiation and Ethyl Methane Sulfonate. The six mutant clones have been tested for stability in the field. They are proven to have high productivity and yields, so that they are very potential to be developed as superior varieties. To support the planting material sufficiency of these clones, an efficient propagation method was needed. Media formulations with different physical properties and composition of growth regulators were tested to obtain high seedling propagation rates. The media formulation for callus induction was Murashige dan Skoog (MS) + 3 mg/l 2,4-D + 3 g/l casein hydrolysate + 3% sucrose and for shoot regeneration was MS + 0.5 mg/l BA + 0.1 mg/l IBA + 100 mg/l PVP and 2% sucrose. Shoot proliferation was carried out on MS liquid (1, ½) + (0.3; 0.5 mg/l) BA + 0.1 mg/l IBA + 1 mg/l Kinetin + (0; 0.5 mg/l) GA3+ sucrose 2%. The results showed that callus induction, callus regeneration, and shoot proliferation of sugarcane mutant clones were influenced by the genotype and medium composition. The fastest callus induction was obtained from the MSP-4 clone (5.82 days), and the longest was MSB-7 (8.82 days). The largest callus diameter was obtained from MSB-6 clone on MS medium containing 1 mg/l BA, 100 mg/l PVP, and 2% sucrose. The highest number of shoots was obtained from the MSB-6 clone, while the least number of shoots conducted from the MSB-8 clone. The MSB-8 clones were more difficult to regenerate compared to the others. The best media formulation for shoot proliferation was ½ MS containing 0.5 mg/l BA, 1 mg/l Kinetin, and 0.1 mg/l IBA, while the best formulation for rooting was ½ MS.

1. Introduction

Sugarcane (Saccharum officinarum L.) is a monocotyledonous crop plant that belongs to the family Poaceae. It is the main crop that supplies sugar and ethanol production. The main byproducts of the sugar industry are bagasse and molasses. Molasses is the main raw material for alcohol and alcohol-based industries. Excess bagasse is also used as a raw material in the paper industry. Indonesia's sugar needs exceed domestic production capacity, so part of it must be met from imports. To achieve this self-sufficiency, providing superior seeds is one of the priorities.

Increasing sugarcane production is influenced by the provision of quality superior seeds. The use of improved varieties has a contribution of 30-35% to yield. Releasing new sugarcane varieties that have superior characteristics must be supported by the propagation of seeds in large quantities to anticipate demand for seeds from newly released varieties. The propagation of sugarcane seeds, which will be
exploited on a large scale in a fast time, will be difficult to achieve through conventional techniques because it suffered from low propagation rates, expensive labor, time-consuming and potential transmission of pathogens through seed cane from generation to generation [1].

Six mutant clones of sugarcane with high productivity have been produced through tissue culture techniques combined with mutations using physical mutagens (gamma-ray irradiation) and chemical mutagens (Ethyl Methane Sulfonate). The six mutant clones have been tested for stability in the field. They are proven to have high productivity and yields, so that they are very potential to be developed as superior varieties. To support the planting material sufficiency of these clones, an efficient propagation method was needed.

Plant tissue culture offers the best methodology through micropropagation of sugarcane for quality and phytosanitary planting material at a faster rate in a shorter period. Propagation of seeds through tissue culture could be scheduled according to the needs and the desired amount, and the level of uniformity of plant material is quite high. According to [2], sugarcane from tissue culture has a better growth rate, tillers, stem production, and yields compared to conventional plants.

The multiplication and proliferation of shoots are a crucial stage in the propagation of any species for commercial exploitation, and the most rapid rates are required. The most common additives to standard media are cytokines, usually BA and Kinetin alone or with the combination of low amount auxins like NAA, IBA, and IAA [1]. However, seed propagation through tissue culture influenced many factors, including type and combination of plant growth regulators, explants type, culture medium composition, and genotype [3]. Therefore, it is necessary to optimize the method for each genotype to achieved the seed production target.

The last stage in shoot propagation through tissue culture is transferring plantlets from the laboratory to the greenhouse (acclimatization). The factor that determines the success of plantlet acclimatization is the root conditions of the plantlets. Plantlets that have perfect roots will greatly support the success of obtaining quality seeds.

2. Methods

2.1 Induction of indirect adventitious shoots and callus regeneration

Plant material (explants) used in this study were young leaves from 6 mutant clones. The young leaves used were rolled leaves isolated from the shoots of sugarcane. The leaves washed using a detergent solution mixed with 30% NaOCl. Then sugarcane shoots were put into Laminar Air Flow (LAF) for further sterilization. The sugarcane shoots are then dipped in 96% alcohol, burned over a fire, and cut into ± 2 mm in a sterile petri dish. After that, the slices of sugarcane leaves are inserted into the growing medium.

The first stage of this activity is callus induction from young leaves. The media formulations used for callus induction were Murashige and Skoog (MS) basic media + 3 mg / l 2,4-D + 3 g / l Casein Hydrolyzate + 3% sucrose. Each bottle contains 2 explants with 20 repetitions. Callus was induced in dark conditions in the culture room at a temperature of 22 ± 2°C...

The callus obtained was transferred to shoot regeneration media, namely MS with the addition of BA (0.5 and 1 mg/l), thidiazuron (0 and 0.1 mg/l), IBA 0.1 mg / l, PVP 100 mg / l, and 2% sucrose) . The cultures were incubated in the culture room at 22°C. Irradiation is carried out for 16 hours in light conditions and 8 hours in dark conditions with a radiation intensity of 1000 lux. The variables observed were callus initiation time, callus percentage formed, callus diameter, and callus color.

2.2 Shoot proliferation using liquid media

Clones with a good growth response were transferred to the media for induce shoot proliferation using two (2) media formulations, namely: 1) MS (liquid) with the addition of 3 mg/l BA, 1 mg/l kinetin, 0.5 mg/l GA3, 2% sucrose, and 200 mg l PVP, and 2) ½ MS (liquid) with the addition of 0.5 mg/l BA, 0.1 mg/l IBA, 100 mg/l PVP and 2% sucrose.
The experimental design used was a completely randomized design with 20 replications. The bottles were placed in a culture rack using a TL lamp with an irradiation intensity of 1000 lux for 16 hours a day at a temperature of 22°C. Culture was incubated statically. Irradiation carried out for 16 hours. Observations were made on the number of shoots produced.

2.3 Rooting induction
Root induction were performed on MSP-2 and MSB-6 clones using MS1/2 + IBA (0 and 0.1 mg / l) media. The experimental design used was a completely randomized design with 20 replications.

The experimental design used was a completely randomized design with 20 replications. The bottles were placed in a culture rack using a TL lamp with an irradiation intensity of 1000 lux for 16 hours a day at a temperature of 22°C. Observations were made on the number of roots formed.

2.4 Data analysis
Data were analyzed using ANOVA at the 95% confidence level. If there is a significant difference between treatments, then a further test is carried out using the Duncan test at the 95% confidence level.

3. Result and discussion

3.1 Induction of indirect adventitious shoots and callus regeneration
The use of MS + 3 mg/l 2,4-D + 3 g/l casein hydrolyzate + 3% sucrose induced callus formation in all clones, with varying responses in all observed variables (Table 1). 2,4-D is a type of auxin with high activity, effective for sugarcane callus induction [4]; [5]. Growth regulators from the auxin group play a role in the callus formation process. At the same time, the selection of types and concentrations is determined by the type of growth and development of the explants, and the genotype used [5], [6]; [7]). The research of [2] showed that 2.4-D with a concentration of 1-6 mg/l was effective in inducing sugarcane callus formation. The addition of casein hydrolyzate to the media containing auxins seems to increase embryogenic callus formation [8]. Casein hydrolyzate is a precursor in the formation of nucleic acids and other cellular processes.

The research showed that the MSP-4 clones could form callus in the fastest time (5.82 days) than other mutant clones, while the callus from MSB-7 and MSB-8 clones were formed the latest at 8.82 and 8.03 days, respectively. MSP-4 clones were also able to produce the highest callus formation, namely 86.13%, while the callus diameter of the six clones was almost the same (Table 1). The callus color of each clone was different. The callus, which is black in color, indicates that the ability of callus proliferation is low (Figure 1).

| Mutant clones | Callus induction (days) | Callus formation (%) | Diameter of callus (cm) | Color         |
|---------------|-------------------------|----------------------|-------------------------|---------------|
| MSP-2         | 6.49                    | 47.50                | 1.08                    | Reddish white|
| MSP-3         | 6.18                    | 50.74                | 1.28                    | Brown         |
| MSP-4         | 5.82                    | 86.13                | 1.73                    | Reddish       |
| MSB-6         | 6.69                    | 65.92                | 1.78                    | Yellowish white|
| MSB-7         | 8.82                    | 36.43                | 1.62                    | Black         |
| MSB-8         | 8.03                    | 37.94                | 1.64                    | Black         |
Figure 1. Callus appearance of six clones of mutant sugarcane.

a = MSP-2, b= MSP-3, c=MSP-4,
d= MSB-6, e = MSB-7, f = MSB-8

TP1, TP2, TP3, and TP4 media influenced callus development until callus regeneration formed shoots. The statistical analysis results showed an interaction between the media formulations and the mutant clones on callus diameter (Table 2). The MSB-6 mutant clones produced the largest callus diameter in all media formulations. In contrast, in the MSB-8 clones, the best callus development was obtained using TP2 and TP3 media, indicated by callus diameters of 2.40 cm and 2.0 cm. The smallest callus diameter resulted from MSP-3 clones.

Table 2. Diameter of callus on media containing cytokinins, 5 weeks after planting

| Mutant clones | TP1    | TP2    | TP3    | TP4    |
|---------------|--------|--------|--------|--------|
| MSP-2         | 1.92 cdefg | 1.92 cdefg | 2.05 bede | 1.63 ghi |
| MSP-3         | 1.40 ij  | 1.25 j  | 1.44 hij | 1.42 ij |
| MSP-4         | 1.63 ghi | 2.02 bcd ef | 1.83 defg | 1.83 defg |
| MSB-6         | 2.22 abc | 2.40 a  | 2.19 abc | 2.12 abcd |
| MSB-7         | 1.69 fghi| 1.61 ghi | 1.47 hij | 1.76 efgh |
| MSB-8         | 1.94 cdefg| 2.30 ab | 2.00 bcd ef | 1.94 cdefg |

Means within a column having the same letter are not statistically significant (p=0.05) according to Duncan’s Multiple Range Test.

TP 1 = MS+ BA 0.5 mg/l + PVP 100 + Sukrosa 2%
TP 2 = MS+ BA 1 mg/l + PVP 100 + Sukrosa 2%
TP 3 = MS+ BA 0.5 mg/l + TDZ 0.1 mg/l + PVP 100 + Sukrosa 2%
TP 4 = MS+ BA 1 mg/l + TDZ 0.1 mg/l + PVP 100 + Sukrosa 2%

The TP2 media formulation appears to be the best medium for callus development. Increasing BA concentration from 0.5 mg/l (TP1) to 1 mg/l (TP2) could increase callus development. However, BA 1 mg/l combined with thidiazuron 0.1 mg/l (TP4) suppresses callus development. The use of BA as a growth regulator in the in vitro propagation of sugarcane has also been reported by [9].

The ability of shoot regeneration varied depending on the plant genotype. MSB-6 clones produced the highest number of shoots on TP2 media (21.6 shoots), followed by MSP-2 on TP1 media (16.50 shoots), while MSP-3 and MSB-8 clones produced the least number of shoots. In all media formulations (Table 3). The results indicated that each clone had a different response to the same media formulation.
This condition indicates that each mutant clone has changed its genetic trait even though all the mutant clones come from the same parent (variety). Changes in genetic traits are due to the treatment given at the time of culture, which causes changes in the DNA arrangement that makes the gene expression is different. These various responses are in accordance with the statement of [11] that genotype affects the growth and development of a plant species.

### Table 3. The number of shoots of six mutant clones in four media formulations, 5 weeks after planting

| Mutant clones | Media | TP1   | TP2   | TP3   | TP4   |
|---------------|-------|-------|-------|-------|-------|
| MSP-2         |       | 16.50 ab | 11.50 bcde | 12.83 bc | 6.67 cdef |
| MSP-3         |       | 3.83 ef  | 2.00 f  | 3.60 ef | 2.20 f  |
| MSP-4         |       | 1.00 f   | 1.30 f   | 3.80 ef | 5.00 def |
| MSB-6         |       | 12.22 f  | 21.60 a  | 16.60 ab | 13.00 bc |
| MSB-7         |       | 5.78 f  | 4.67 def | 1.22 f  | 2.30 f  |
| MSB-8         |       | 1.10 f   | 1.10 f   | 1.10 f  | 1.60 f  |

Means within a column having the same letter are not statistically significant (p=0.05) according to Duncan’s Multiple Range Test.

TP 1 = MS + BA 0.5 mg/l + PVP 100 + Sukrosa 2%
TP 2 = MS + BA 1 mg/l + PVP 100 + Sukrosa 2%
TP 3 = MS + BA 0.5 mg/l + TDZ 0.1 mg/l + PVP 100 + Sukrosa 2%
TP 4 = MS + BA 1 mg/l + TDZ 0.1 mg/l + PVP 100 + Sukrosa 2%

Sugarcane callus production and regeneration depend on the plant genotype, the source of the explants used, and the media formulation to regenerate it [5]; [9]; [12]; [13]. Plant regeneration through tissue culture is usually specific, which means that media formulations that can be used to regenerate certain plant varieties may not necessarily be used for other varieties [8].

The callus regeneration ability to form shoots was not affected by the callus induction rate of each mutant clone. MSP-4 clones were able to form a callus at the fastest time than MSP-2 and MSB-6 clones (Table 1), but callus was not able to divide and form shoots rapidly (Table 2 and 3). On the other hand, MSP-2 and MSB-6 clones, although initially, the callus formation was rather slow, the callus could develop well, even the fastest shoot formation. The results obtained indicated that the regeneration ability of each clone was different.

### Table 4. The number of shoots from two mutant clones in shoot proliferation, 4 Weeks After Planting

| Media (liquid) | MSP-2 | MSB-6 |
|---------------|-------|-------|
| MS + BA 0.3 + K1 + IBA0.1 + GA3 0.5+S2% (P1) | 9.80 abc | 7.00 abc |
| ½ MS + BA 0.5 + K1+ IBA0.1+S2% (P2)   | 11.00 ab | 14.67 a |

Means within a column having the same letter are not statistically significant (p=0.05) according to Duncan’s Multiple Range Test.

Statistical analysis showed no significant effect of the two media formulations used on the number of shoots produced. The highest number of shoots was produced from MSB-6 clones on P2 media (14.67 shoots), as well as in MSB-2 clones (11 shoots) (Table 4). The use of liquid media does not appear to increase the proliferation of shoots of sugarcane. This is different from the research by[14], which
showed that the use of MS media containing BA 0.5 mg/l and IBA 0.1 mg/l could increase the number of shoots produced.

Figure 2. Shoots proliferation on liquid media.

3.2 Root induction
Rooting is one of the stages in plant propagation that determines the transfer of plant tissue culture products (plantlets) to the greenhouse (acclimatization). Plants with many roots with root hairs are needed for plantlet adaption in the greenhouses.

The statistical analysis results showed no interaction between clones and the formulations used on sugarcane rooting. The number of roots produced was not significantly different between R1 and R2 media, namely 13.50 and 16.20, respectively (Table 5). Similar results were obtained from the number of roots in the MSP-2 and MSB-6 clones (Table 6).

The use of auxin growth regulators (NAA and IBA) is effective in accelerating the initiation of the root formation of sugarcane. The use of IBA 1 mg/l was able to induce rooting of sugarcane by 90% [10]. However, in this study, the use of ½ MS medium without the addition of IBA was able to induce rooting in sugarcane clones to reduce production costs. Different results were also shown from the study of [15], which showed that the use of 2.5 mg/l NAA on MS medium stimulated root formation.

| Media formulation | 2 MST | 3 MST | 4 MST |
|-------------------|-------|-------|-------|
| ½ MS + IBA 0.1 (R 1) | 4.6 a | 9.9 a | 13.50 a |
| ½ MS (R 2) | 5.0 a | 12.6 a | 16.20 a |

Means within a column having the same letter are not statistically significant (p=0.05) according to Duncan’s Multiple Range Test

Table 6. Roots number of two mutant clones, 4 weeks after planting.

| Mutant clones | 2 MST | 3 MST | 4 MST |
|---------------|-------|-------|-------|
| MSP-2 | 5.25 a | 12.4 a | 17.45 a |
| MSB-6 | 4.60 a | 10.63 a | 14.58 a |

Means within a column having the same letter are not statistically significant (p=0.05) according to Duncan’s Multiple Range Test
4. Conclusion
Callus induction, callus regeneration, and shoot proliferation of sugarcane mutant clones in vitro were influenced by the media's genotype and composition. The fastest callus induction came from the MSP-4 mutant clone, which was 5.82 days, and the longest was MSB-7, which was 8.82 days. The largest callus diameter was obtained from MSB-6 clones on MS medium containing BA 1 mg/l, PVP 100 mg/l, and sucrose 2%. The highest number of shoots was obtained from MSB-6 clones, while the least number of shoots were conducted from MSB-8 clones. MSB-8 clones tend to have a difficult response to regenerate than other clones. The best media formulation for shoot proliferation was ½ MS containing 0.5 mg/l BA, 1 mg/l Kinetin and 0.1 mg/l IBA, while the formulation to induce rooting was ½ MS.

References
[1] Getnet B 2017 Review On In Vitro Propagation of Sugarcane to Advance The Value of Tissue Culture. Agric. Res, and Technol, 54 99-104
[2] Jalaja N C, Neelamathi D and Sreenivasan T V 2008 Micropropagation for Quality Seed Production in Sugarcane in Asia and The Pacific (FAO, APCoAB and APAARI) 46
[3] Hailu M, Chimdessa M and Muthswamy M 2018 In Vitro Propagation of Selected Sugarcane (Saccharum officinarum L.) Varieties (C 86-165 and C 86-12) Through Shoot Apical Meristem. Int. J Hort & Agric. www.symbiosisonline.com
[4] Gandonou C, Errabii T, Abrini J, Idaomari M, Chibi F and Senhaji N S 2005 Effect of Genotype on Callus Induction and Plant Regeneration from Leaf Explants of Sugarcane (Saccharum sp.). African J. Biotechnol 411 1250-55
[5] Ali A, Naz S, Siddiqui F A and Iqbal J 2008 Rapid Clonal Multiplication of Sugarcane (Saccharum officinarum) Through Callogenesis and Organogenesis. Pak. J. Bot. 411 123-138
[6] George F E, Hall M A and Geert-Jan De Klerk 2008 Plant Propagation by Tissue Culture. The Background. (Springer Publisher. Dordrecht, Netherlands). 501 p
[7] Jamil S, Shahzad R, Talha G M, Sakhawat G, Rahman S U, Sultana R dan Iqbal M Z 2017 Optimization of Protocols for In Vitro Regeneration of Sugarcane (Saccharum officinarum). Int. J. Agronomy 1 1-8
[8] Purnamaningsih R 2006 Induksi Kalus dan Optimasi Regenerasi Empat Varietas Padi Melalui Kultur In Vitro J. AgroBiogen 22 74-80
[9] Sukmadjaja D, Supriati Y dan Pardal S J 2014 Kultur Apeks untuk Penyediaan Bibit Unggul Tebu Varietas PS864 dan PS881. J AgroBiogen 102 45-52
[10] Azizi AAA, Roostika I dan Efendi D 2017 Multiplikasi Tunas In Vitro Berdasarkan Jenis Eksplan pada Enam Genotipe Tebu (Saccharum officinarum L.). J LITTRI. 232 90-7
[11] Ali S, Iqbal J and Khan M S 2010 Genotype Independent In Vitro Regeneration System in Elite
Varieties of Sugarcane *Pakistan J of Bot.* 426 3783-90

[12] Behera K K and Sahoo S 2009 Rapid In Vitro Micropropagation of Sugarcane (*Saccharum officinarum* L. cvNayana) Through Callus Culture *Nat. Sci* 74 1-10

[13] Sukmadjaja D dan Mulyana A 2011 Regenerasi dan Pertumbuhan Beberapa Varietas Tebu (*Saccharum officinarum* L.) secara *in vitro.* *J Agro Biogen* 72 106-18

[14] Roostika I, Rahayu S, Sukmadjaja D dan Mariska I 2015 Aplikasi Teknik Kultur Cair Untuk Efisiensi Perbanyakan Tunas Tebu Secara Kultur Jaringan. *Pros., Sem., Nas., Tebu Inovasi Teknologi Budi Daya Tebu Mendukung Swasembada Gula,* Malang 22 April 2014 Balittas. 248 hal

[15] Dinesh P, Thirunavukkarasu P, Saraniya A R and Ramanathan T 2015 In Vitro Studies of Sugarcane Variety Co-91017 Through Micropopagation of Shoot Tip Culture. *Adv Plants Agric Res* 26:263–68