THE EFFECT OF PH AND SUCROSE ON THE EMBRYOGENIC CELLS GROWTH OF SUGAR CANE (*Saccharum officinarum*) IN LIQUID CULTURE †

[EL EFECTO DEL PH Y LA SACAROSA EN EL CRECIMIENTO DE CÉLULAS EMBRIOGÉNICAS DE CAÑA DE AZÚCAR (*Saccharum officinarum*) EN CULTIVO LÍQUIDO]

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SUMMARY

Background. Sugarcane (*Saccharum officinarum* L.) is one of the important commodities in Indonesia as raw material for sugar production. The constraints for sugarcane cultivation are limited availability and poor quality of seedling. The utilization of liquid culture in tissue culture enables to produce good quality seedlings, free of viruses and diseases, and can generate a large quantity in a short time. Liquid culture can produce embryogenic cells faster since embryogenic callus can develop easily in liquid media. **Objective**. This study determined the appropriate pH and concentration of sucrose in the liquid culture of embryogenic cells. **Methodology**. This study was conducted in 3 stages of in vitro culture, which were callus induction, solid media proliferation, and liquid media proliferation that using pH and sucrose treatments. **Result**. The results showed that pH 6.5 + 3% sucrose (A3B1) produced the highest number of callus with the average callus on the first week 29 callus, the second week 8,33 callus and the third week 4,33 callus and the color did not easily turn to brown with color 5Y 8/2. **Implications**. The protocol was developed that allows embryogenic callus of sugarcane plants to be obtained in liquid media that can be used for embryogenic callus proliferation. **Conclusions**. pH 6.5 + sucrose 3% (A3B1) was the best combination treatment of pH and sucrose for sugarcane callus proliferation in liquid culture media, which produced the highest number of calluses and color of callus that not easily turned brown.
Keywords*:* somatic embryogenesis; callus; proliferation; globular; scutellar.

RESUMEN

Antecedentes. La caña de azúcar (Saccharum officinarum L.) es uno de los productos básicos más importantes en Indonesia como materia prima para la producción de azúcar. Las limitaciones para el cultivo de la caña de azúcar son la disponibilidad limitada y la mala calidad de las plántulas. La utilización del cultivo líquido en el cultivo de tejidos permite producir plántulas de buena calidad, libres de virus y enfermedades, y puede generar una gran cantidad en poco tiempo. El cultivo líquido puede producir células embriogénicas más rápidamente ya que el callo embriogénico puede desarrollarse fácilmente en medios líquidos. **Objetivo**. Este estudio determinó el pH y la concentración de sacarosa adecuados en el cultivo líquido de células embriogénicas. **Metodología**. Este estudio se llevó a cabo en 3 etapas de cultivo in vitro, que fueron la inducción de callo, la proliferación en medio sólido y la proliferación en medio líquido que utilizando tratamientos de pH y sacarosa. **Resultados**. Los resultados mostraron que el pH 6,5 + 3% de sacarosa (A3B1) produjo el mayor número de callos con el promedio de callos en la primera semana 29 callos, la segunda semana 8,33 callos y la tercera semana 4,33 callos y el color no se convirtió fácilmente en marrón con el color 5Y 8/2. **Implicaciones**. Se desarrolló un protocolo que permite obtener callos embriogénicos de plantas de caña de azúcar en medios líquidos que pueden ser utilizados para la proliferación de callos embriogénicos. **Conclusiones**. pH 6,5 + sacarosa 3% (A3B1) fue el mejor tratamiento de combinación de pH y sacarosa para la proliferación de callos de caña de azúcar en medios de cultivo líquidos, que produjo el mayor número de callos y el color del callo que no se volvió marrón fácilmente.
Palabras Clave: embriogénesis somática; callo; proliferación; globular; escutelar.

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INTRODUCTION

Sugarcane (*Saccharum officinarum* L.), a raw material for sugar production is one of the important commodities in Indonesia. According to The Directorate General of Plantation (2019), sugarcane production in Indonesia tends to decrease from 2011 until 2019. According to Sulaiman *et al* (2019), the amount of sugar consumption increases by 4.3% every year which made Indonesia the largest sugar importer in 2017-2018. This problem occurred because sugarcane production was unable to meet the needs of raw materials for sugar production due to low availability and poor quality of seedlings. Provision of seedlings can be conducted conventionally or unconventionally by tissue culture.

Tissue culture is a method for isolating part of a plant and maintaining cells or pieces of plant tissue grown on suitable artificial media under aseptic conditions (Nofrianinda *et al*., 2017). Tissue culture technique is classified into organogenesis and somatic embryogenesis. Organogenesis is the process of direct organ formation from explants (Hinchee *et al*., 1988). While somatic embryogenesis is a regeneration process by forming an embryo-like structure derived from somatic cells with plumule and radicle (Pardal, 2003). The advantages of somatic embryogenesis are shorter propagation times, bipolar embryo production, higher transformation ability, and can be utilized for germplasm storage (Sapsuha *et al*., 2011). Somatic embryogenesis can be developed using solid or liquid media. An embryogenic callus is easier to develop using liquid media because it accelerates the growth of cells (Taryono, 2016). According to Minarsih *et al* (2013), temporary immersion system method in liquid culture can produce higher number and uniformity shoots and plantlets compare to solid media.

Liquid culture is the culture of free cells or small cell aggregates in a liquid medium by shaking method (Gunawan, 1992). The success of liquid culture is influenced by explants (cultured plant parts), hormones, media composition, the physical environment of tissue culture, and growth regulators. The pH and sucrose contents during proliferation are media composition factors affecting the growth of callus. The pH of medium indicates the value of acidity and alkalinity of a solution in water. It indicates the presence of H+ ions in the solution and affects the solubility of compounds in form of salt (Mastuti, 2017). According to Mishra *et al* (2019), pH media of 5.82 produced high biomass and alkaloids. Sucrose is a source of carbon in tissue culture. Disaccharides application in the form of sucrose will provide an optimum growth response in tissue culture (Azmi *et al*, 2017). Furthermore, the application of 5% sucrose can increase the production of cell biomass and alkaloid content in *Eurycoma longifolia* cell (Siregar, 2010). This finding also conveyed by Hanifa (2013) that higher sucrose concentration increased the dry weight of *Croton tiglium*. Meanwhile, according to Yaacob *et al* (2014), the highest callus production obtained in sucrose concentration by 5%, but the fastest callus growth (25-30 days) exhibited in 3% sucrose application. Tissue culture media with pH 5.8 and 6.8 was proven to induce callus growth within 30 days in lime. Therefore, it is necessary to evaluate the effectiveness of pH and sucrose application in liquid culture media for enhancing the growth of embryogenic cells of sugarcane.

MATERIALS AND METHODS

The study was conducted at the Center for Development of Advanced Sciences (CDAST) Laboratory in University of Jember from April 2020 to May 2021. The plant material used was obtained from the spindle leaf of Sugarcane "Bululawang" (BL) variety. Plant material was taken from 4-6 months old healthy plants in the field. The spindle leaf was prepared and sterilized with 70% alcohol and placed in Laminar Air Flow (LAF) cabinet. The plant tip dipped in 96% alcohol, and then the whole plant material was heated over a bunsen burner to minimize contamination. The sterilized spindle leaf peeled to a 5 mm diameter and sliced into ± 2 mm width on filter paper then cut into 7 pieces.

Callus Induction

These entire experiments using Murashige and Skoog (MS) media as the based media with addition of plant growth regulators for each stage of culture. Callus induction was carried out by planting explants in MS + 4 mg L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) + 300 mg L^{-1} Casein Hydrolysate + 30 g L^{-1} sucrose + 5 g L^{-1} agar with a pH of 6.2. The explants were planted in LAF and the culture bottles were stored on a culture rack for 6 weeks at a temperature of 23°-25°C in a dark room. The explants were sub-cultured every 3 weeks. The induced callus was selected and the embryogenic callus sub-cultured into solid proliferation media. Morphological observations using a Leica EZ4HD microscope.

Solid Media Callus Proliferation

The 6 weeks old callus transferred to solid proliferation media for 3 weeks. The selected callus was embryogenic callus characterized with crumb texture and white or yellowish white color. Proliferation was carried out in dark conditions. Callus proliferation used MS + 2,4-D 2 mg L^{-1} + 500 mg L^{-1} Casein Hydrolysate + 560 mg L^{-1} L-Prolin + 40 mg L^{-1} L-Glutamine + 30 g L^{-1} Sucrose + 5 g L^{-1} agar. Morphological observations using a Leica EZ4HD microscope

Liquid Media Callus Proliferation

The 9 weeks old callus was transplanted to the combination of pH (5.5; 6.0; 6.5) and sucrose (3%, 4%, 5%) liquid media (Table 1). The media contained MS + 2 mg L^{-1} 2,4-D + 500 mg L^{-1} Casein Hydrolysate + 560 mg L^{-1} L-Proline + 40 mg L^{-1} L-Glutamine + 50 mg L^{-1} Arginine + 300 mg L^{-1} Polyvinylpyrrolidone (PVP). The media was placed into erlenmeyer or bottles then sterilized in an autoclave and incubated for 24 hours. Selected callus from proliferation in solid media were pressed with a scalpel. The selected callus was weighed to determine weight. As much as 0.25 g of callus was put into 25 mL liquid media in a 100 mL Erlenmeyer. The culture media was then shaken using a shaker at 100 rpm in the dark condition and 25°C room temperature. Shaking was carried out for 2 hours, and then left for 6 hours. Shaking was repeated 3 times within 24 hours.

Table 1. The combination of pH and sucrose treatments.

Code	Combination of treatments
A_1B_1	pH 5.5 + sucrose 3%
A_1B_2	pH 5.5 + sucrose 4%
A_1B_3	pH 5.5 + sucrose 5%
A_2B_1	pH 6.0 + sucrose 3%
A_2B_2	pH 6.0 + sucrose 4%
A_2B_3	pH 6.0 + sucrose 5%
A_3B_1	pH 6.5 + sucrose 3%
A_3B_2	pH 6.5 + sucrose 4%
A_3B_3	pH 6.5 + sucrose 5%

Callus proliferation was carried out for 3 weeks and selection of callus was conducted every week during the culture period. The callus selection was carried out 3 times. The liquid callus proliferation was filtered with 1 mm diameter filter to collect the number of selected callus data. The bigger clumps of callus which filtered were counted visually on the sterile petridish. While smaller callus (< 1 mm diameter size) which passed the filter were continue to be shaken for a week before filtered again in the next week.

Callus morphological and color of callus observations were carried out microscopically with a Leica EZ4HD microscope and observation of callus cells using Olympus CX31 microscope. Color observation was conducted using Munsell Color Charts for Plant Tissues (Munsell Color 1977) every week microscopically. The selected callus that filtered was chosen as sample for color of callus data. The selected callus to be observed were callus that had an average color in one callus group and has represented one group of these calluses.

Data Analysis

Two types of data were collected, they were qualitative and quantitative data. Qualitative data which include morphology of callus and color of callus. Meanwhile quantitative data which include number of selected callus. Complete random design (CRD) with 2 factors was used for the experimental design for this research. The quantitative data were analyzed statistically by analysis of variance (ANOVA) using the least significant different (LSD) test ($P<0.05$) in Ms Excel.

RESULTS

Callus Induction

Morphology of callus

Callus development starts from a pre-embryo mass (PEM) which develops into a globular mass. The PEM phase started to appear in the 2nd week after planting which was characterized by the bigger size of explants and appearance of nodular. Pre-embryo mass was characterized by a soft structure and yellowish-white color. The callus began to appear in the 4th week and was visible in the 6th week (Figure 1). The globular stage began to appear at the 6th week, marked by the appearance of protrusions on the callus due to cell division.

Color of callus

Callus color observations are performed using munsell chart. Callus produced at 6th week after planting classified into embryogenic and non-embryogenic callus. Embryogenic callus had a clear white or yellowish-white color, dry and shiny, while non-embryogenic callus was brownish or blackish and wet (Figure 2). Embryogenic callus had color 5Y 8/2, while the callus non embryogenic colored 5Y 5/6.

Callus Proliferation using Solid Media and Liquid Media

The 9 weeks old callus entered the globular stage and some callus had formed the scutellar stage. The following pictures show the difference between embryogenic and non-embryogenic callus in solid proliferation media (Figure 3). The purpose of proliferation using liquid media was for maturity and multiplies of callus. The various pH and sucrose application treatments resulted in different effects (Figure 4).

Morphology of callus

Callus collected from 1 week shaking entered the globular stage and scutellar stage (Figure 5). Callus collected from 2 weeks of shaking started to turn brownish. The callus on A2B1 and A3B1 began the globular stage (Figure 6). The callus collected from

3 weeks of shaking started turning into a brownish color. The average number of callus was 3 to 6. Callus collected from 3 weeks shaking mostly non-embryogenic which color turned into brownish and blackish (Figure 7).

The color of callus

Callus color observations were performed using munsell color chart. The 3% sucrose treatment resulted in a slow-browning callus, while the 4% and 5% sucrose treatments resulted in a fast-browning callus. Treatment of pH 6.5 + 3% sucrose (A3B1) produced the best callus which color did not easily turn brown that shown in Table 2.

On the treatment A1B1 changes the color of the callus became increasingly dark, on the 1st week of color 5Y 8/4 that is yellowish white to change in the 2nd week of being 5Y 7/4 that is brownish-yellow, and in the 3rd week turned into 5Y 6/2 into brown color. While on treatment A3B1 every week does not change color, the color of the callus remains on the 5Y 8/2 namely the white though in the third week there were only little brown spots on the callus.

Number of selected callus

The average callus produced after 1 week of shaking on the combination treatments of pH and sucrose were not significantly different (Figure 8). The highest number of callus was found in pH 6.5 + 5% sucrose (A3B3), which were 29 calluses. Meanwhile, the lowest number of callus was found in pH 6 + 5% sucrose (A2B3), which were 22 calluses. The average callus produced after 2 weeks of shaking on the combination treatments of pH and

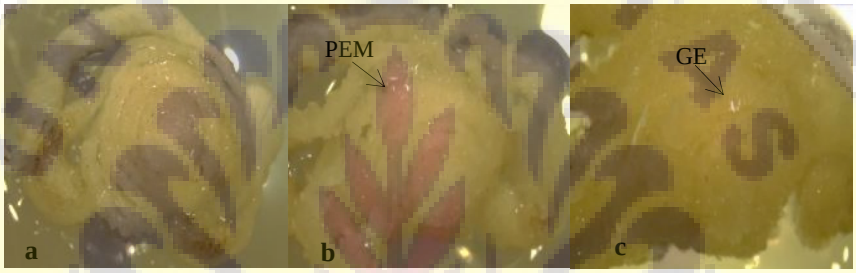

Figure 1. Development of spindle leaf explant of sugarcane on callus induction media. 2 weeks old spindle leaf (a). 4 weeks old spindle leaf (b), 6 weeks old spindle leaf (magnified 8x, scale bars 1 mm) (c). (PEM: pre-embryo mass, GE: Globular embryo).

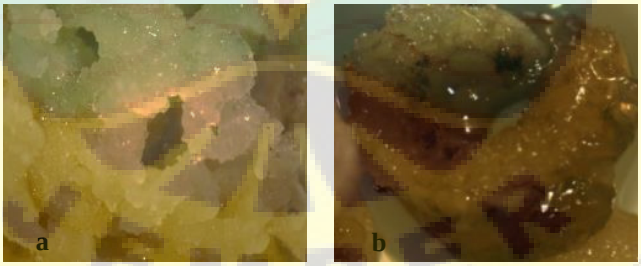

Figure 2. The different colors of embryogenic and non embryogenic callus at the 6th week after planting. Embryogenic callus with white or yellowish white color (a). Non-embryogenic callus with brownish or blackish color (b). (Magnified 8-16x, scale bars 1 mm).

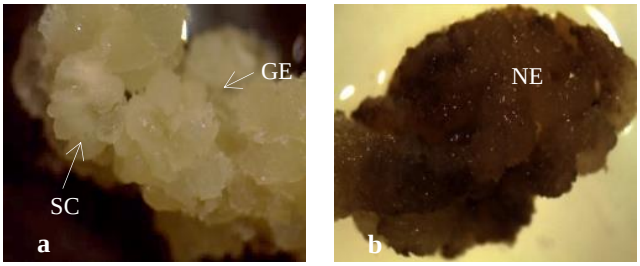

Figure 3. The difference of embryogenic and non-embryogenic callus at 9th week after planting. Embryogenic callus (a). Non-embryogenic callus (b). (Magnified 8-16x, scale bars 1 mm). (GE: Globular embryo, SC: Scutellar, NE: Non-embryogenic).

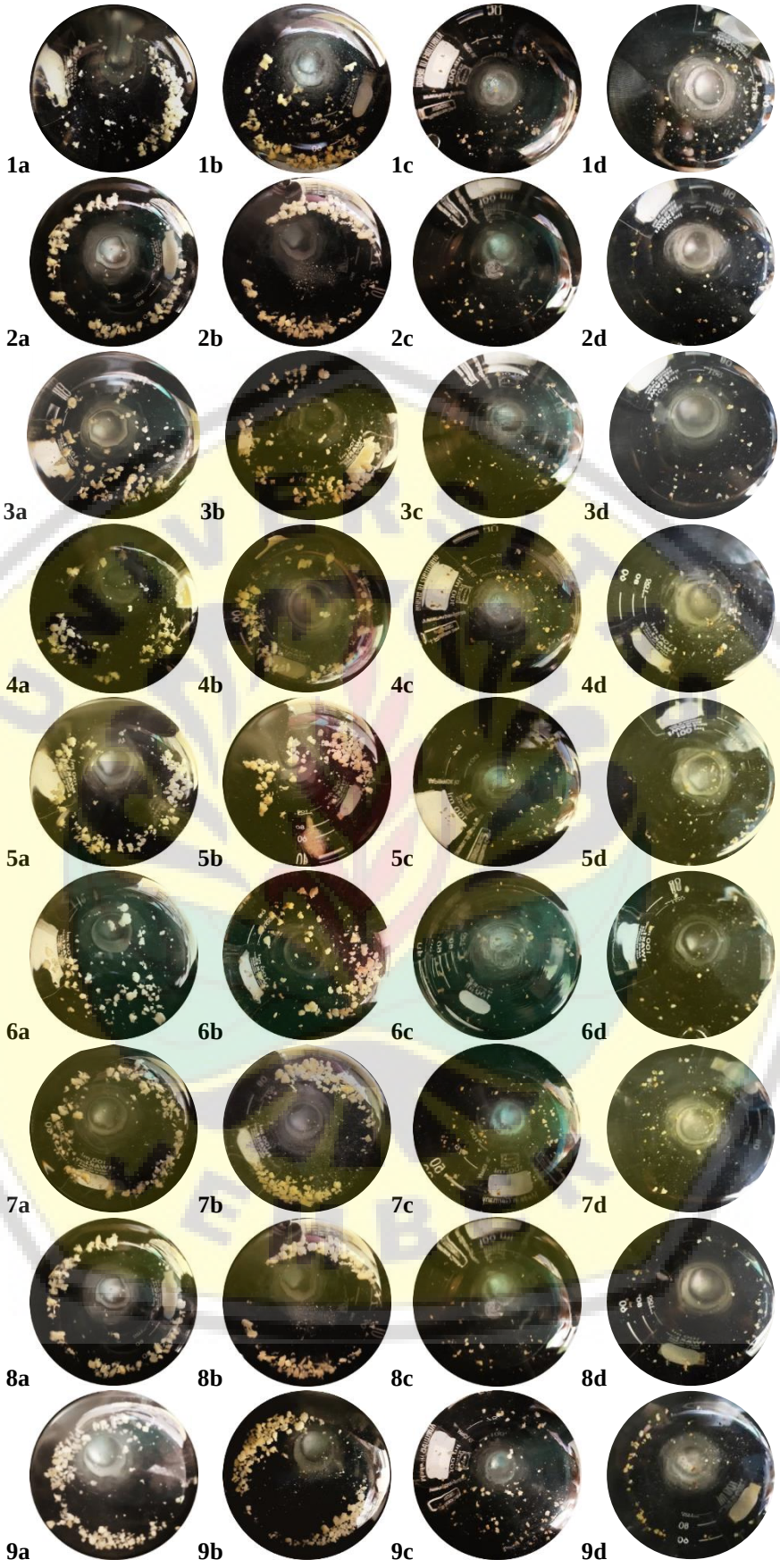

Figure 4. Callus proliferation on MS liquid media. 1) Media A1B1. 2) Media A1B2. 3) Media A1B3. 4) Media A2B1. 5) Media A2B2. 6) Media A2B3. 7) Media A3B1. 8) Media A3B2. 9) Media A3B3. The initiation of culture (a). 1 week shaking (b). 2 weeks shaking (c). 3 weeks shaking (d).

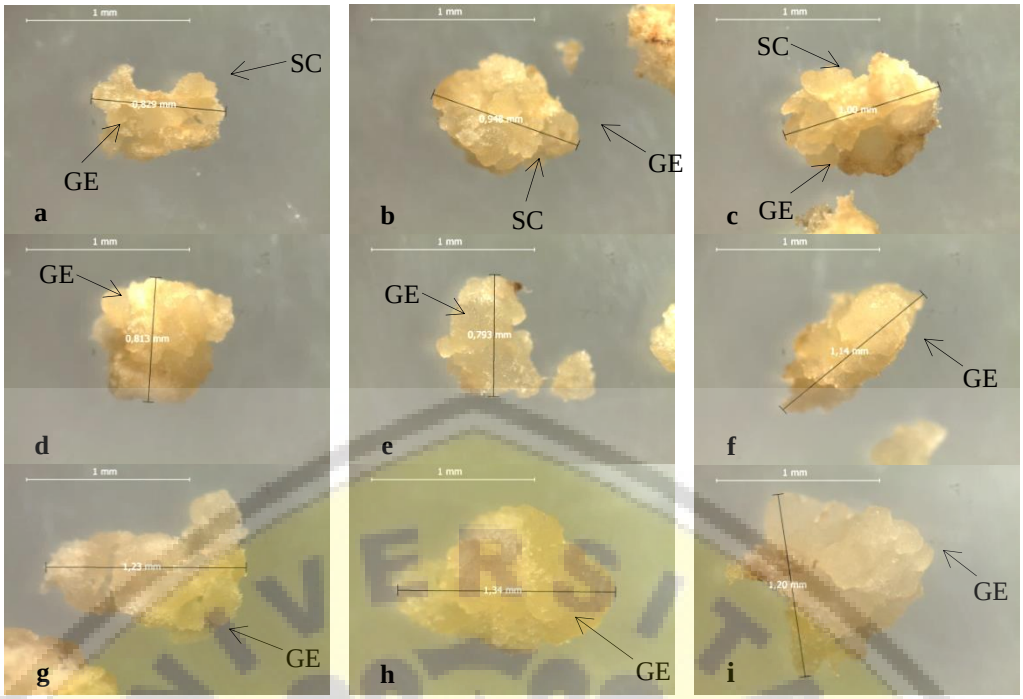

Figure 5. Morphology of callus collected from 1 week shaking. (a) pH 5.5 + sucrose 3% (A1B1). (b) pH 5.5 + sucrose 4% (A1B2). (c) pH 55 + sucrose 5% (A1B3). (d) pH 6.0 + sucrose 3% (A2B1). (e) pH 6.0 + sucrose 4% (A2B2). (f) pH 6.0 + sucrose 5% (A2B3). (g) pH 6.5 + sucrose 3% (A3B1). (h) pH 6.5 + sucrose 4% (A3B2). (i) pH 6.5 + sucrose 5% (A3B3). (Magnified 16-20x, scale bars 1 mm) (GE: Globular embryo, SC: Scutellar).

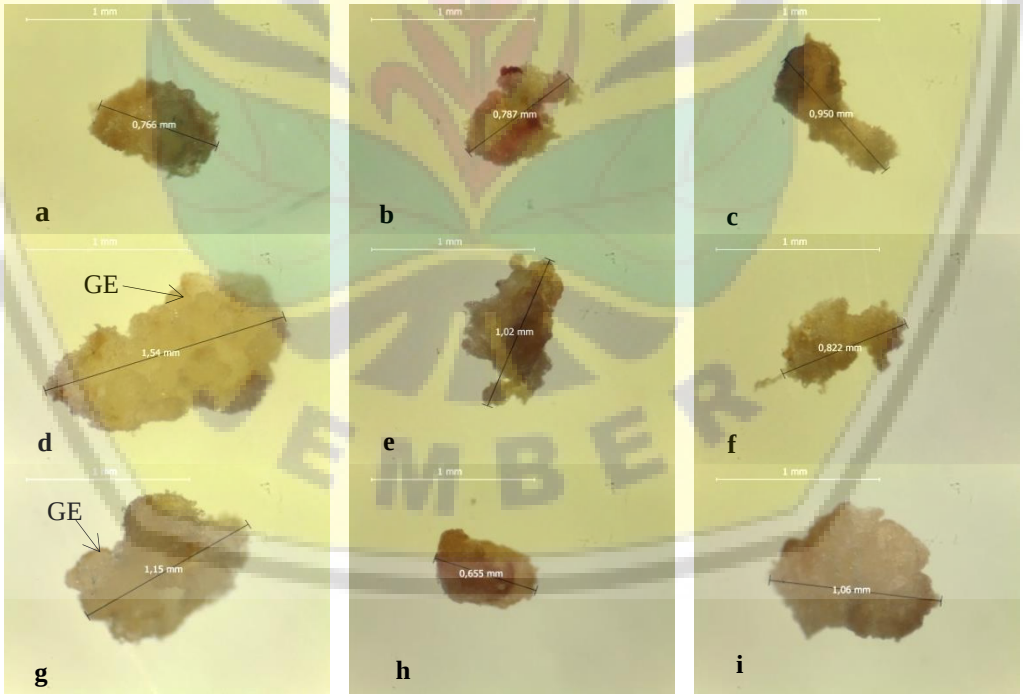

Figure 6. The morphology of callus collected from 2 weeks of shaking. (a) pH 5.5 + sucrose 3% (A1B1). (b) pH 5.5 + sucrose 4% (A1B2). (c) pH 55 + sucrose 5% (A1B3). (d) pH 6.0 + sucrose 3% (A2B1). (e) pH 6.0 + sucrose 4% (A2B2). (f) pH 6.0 + sucrose 5% (A2B3). (g) pH 6.5 + sucrose 3% (A3B1). (h) pH 6.5 + sucrose 4% (A3B2). (i) pH 6.5 + sucrose 5% (A3B3). (Magnified 35x, scale bars 1 mm) (GE: Globular embryo).

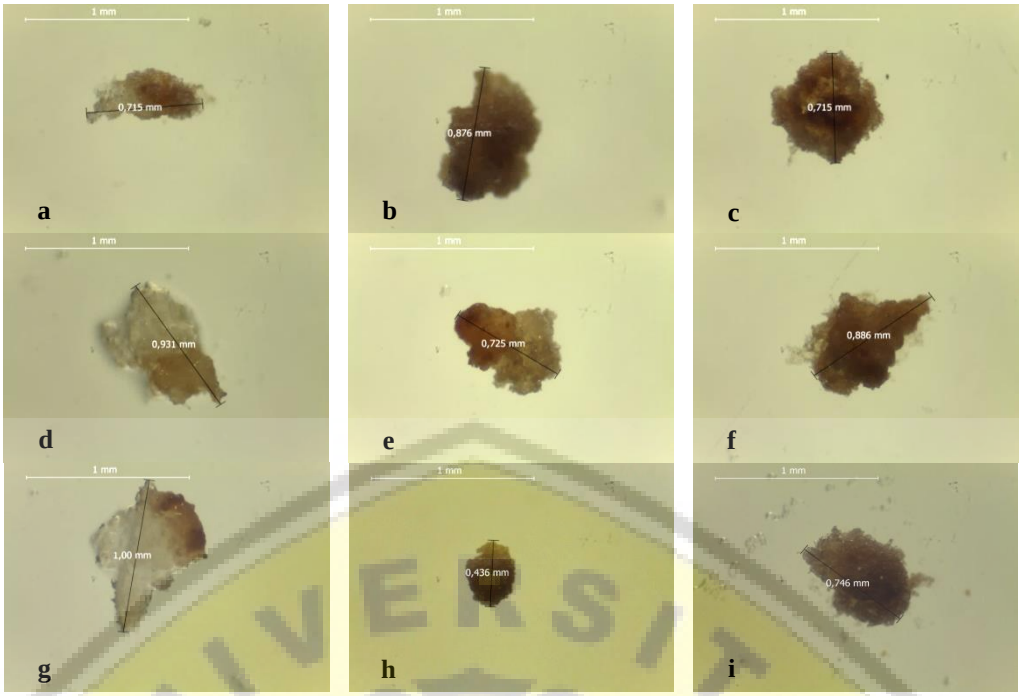

Figure 7. The morphology of callus collected from 3 weeks shaking. (a) pH 5.5 + sucrose 3% (A1B1). (b) pH 5.5 + sucrose 4% (A1B2). (c) pH 55 + sucrose 5% (A1B3). (d) pH 6.0 + sucrose 3% (A2B1). (e) pH 6.0 + sucrose 4% (A2B2). (f) pH 6.0 + sucrose 5% (A2B3). (g) pH 6.5 + sucrose 3% (A3B1). (h) pH 6.5 + sucrose 4% (A3B2). (i) pH 6.5 + sucrose 5% (A3B3). (Magnified 35x, scale bars 1 mm).

Table 2. Callus colors on MS liquid proliferation media.

No	Treatment	1st week	2nd week	3rd week
a	A_1B_1	5Y 8/4 Yellowish white	5Y 7/4 Brownish yellow	5Y 6/2 Brownish
b	A_1B_2	5Y 8/4 Yellowish white	5Y 7/4 Brownish yellow	5Y 5/2 Blackish
c	A_1B_3	5Y 8/4 Yellowish white	5Y 6/4 Brownish +	5Y 5/2 Blackish
d	A_2B_1	5Y 8/4 Yellowish white	5Y 8/2 White	5Y 8/4 Yellowish white
e	A_2B_2	5Y 8/4 Yellowish white	5Y 5/2 Blackish	5Y 6/4 Brownish +
f	A_2B_3	5Y 8/4 Yellowish white	5Y 5/2 Blackish	5Y 5/4 Blackish +
g	A_3B_1	5Y 8/2 White	5Y 8/2 White	5Y 8/2 White
h	A_3B_2	5Y 8/2 White	5Y 6/4 Brownish +	5Y 5/2 Blackish
i	A_3B_3	5Y 8/2 White	5Y 8/4 Yellowish white	5Y 5/2 Blackish

sucrose were not significantly different. The highest number of callus was found in pH 6.5 + 3% sucrose (A3B1), which were 8 calluses. Meanwhile, the lowest number of callus was found in pH 6.0 + 5% sucrose (A2B3), which were 4 calluses. The average callus produced after 3 weeks of shaking on the combination treatments of pH and sucrose were not significantly different. The highest number of callus was found in pH 6.5 + 5% sucrose (A3B3), which were 6 calluses. Meanwhile, the lowest number of callus was found in pH 6.0 + 3% sucrose (A2B1), which were 3 calluses.

Callus cell

The observation of callus cells was conducted at the end of the shaking period. Observations were using an Olympus CX31 microscope with a magnification of 4-10x. The obtained cells were classified into embryogenic and non-embryogenic cells. Embryogenic cells were characterized by round shapes with a nucleus, while non-embryogenic cells have an elongated shape (Figure 9). According to Sajid *and* Aftab (2016) the embryogenic cell shape in sugarcane is characterized by the smaller size

cell size and grow in groups, while non-embryogenic cell characterized by elongated shape.

DISCUSSION

Callus obtained from the 6 weeks induction by planting the spindle leaf explants. Induced callus developed every week starting from the pre-embryo mass (PEM) stage until the globular phase (Figure 4). According to Busaifi & Hirjani (2018), the appearance of callus can be observed by the presence of swelling in the incision that forms small circles. Callus appeared at the 2nd week of the incubation period and was characterized by the appearance of small, clear white circles on the former slice part. PEM is an actively dividing tissue developed from groups of cells that undergo elongation and vacuolation with globular embryos. PEM has the potential to produce somatic embryos (Damayanti *et al*., 2015). Callus formation is influenced by the presence of growth regulators and environmental conditions. Explants with low endogenous content require additional exogenous auxin to trigger callus formation (Astutui *et al*, 2019).

The number of embryogenic callus was obtained higher than non-embryogenic callus which was indicated by the difference in the morphology and color of callus. The 2,4-D growth regulator can initiate callus formation on sugarcane spindle leaf and is commonly used in cell suspension for its ability to support morphogenesis optimally (Sudrajat *et al*., 2016). A higher concentration of 2,4-D can stimulate cell division and assist endogenous auxin to accelerate callus formation. Callus induction using 4 mg L^{-1} 2,4-D can produce the highest number of callus (Sholeha *et al*., 2015; Maulidiya *et al*., 2020). Embryogenic callus is characterized by white or yellowish-white color, crumbly texture, and dry. The crumble texture of callus is caused by a large amount of intercellular space. Meanwhile, non-embryogenic callus was brownish or even blackish in color, compact texture, and wet. The compact texture is caused by very tight cells and difficult to separate (Fiah *et al*., 2014). Non-embryogenic callus changed the color to brown (browning). The cause of browning is a high concentration of phenolic compounds that inhibit the growth of explant (Astuti *et al*., 2019).

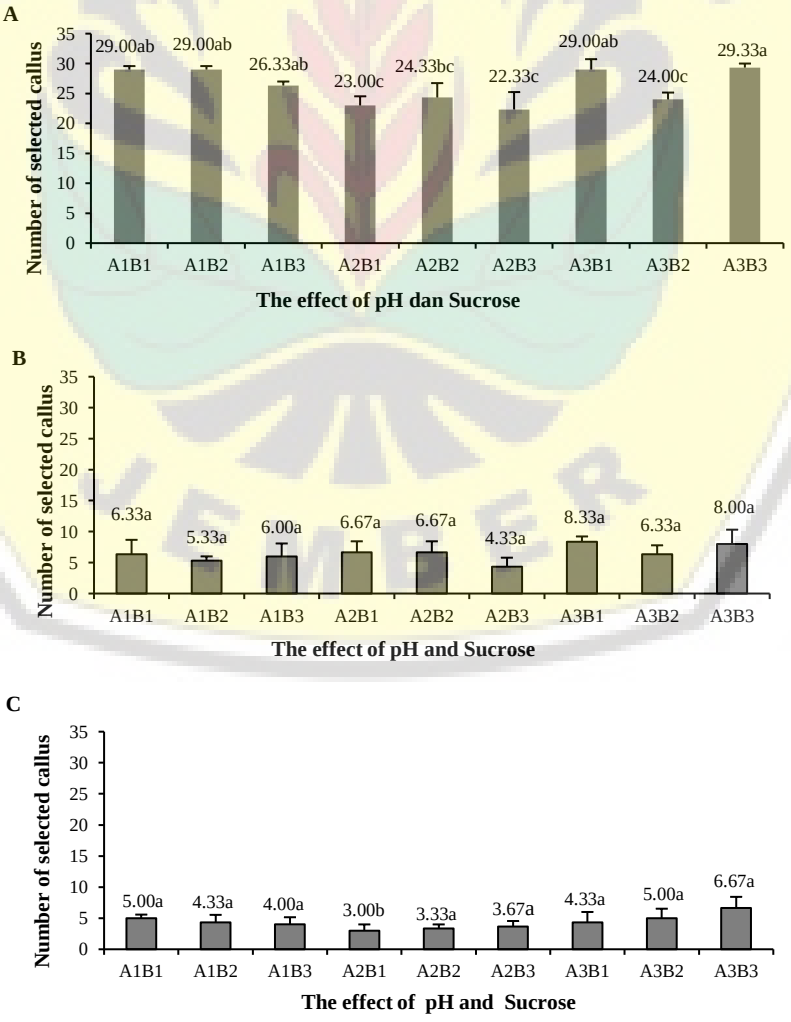

Figure 8. Number selected callus after planting on liquid media. (A). at 1 week. (B). at 2 weeks. (C). at 3 weeks. Values followed by a different letter differ at P<0.05.

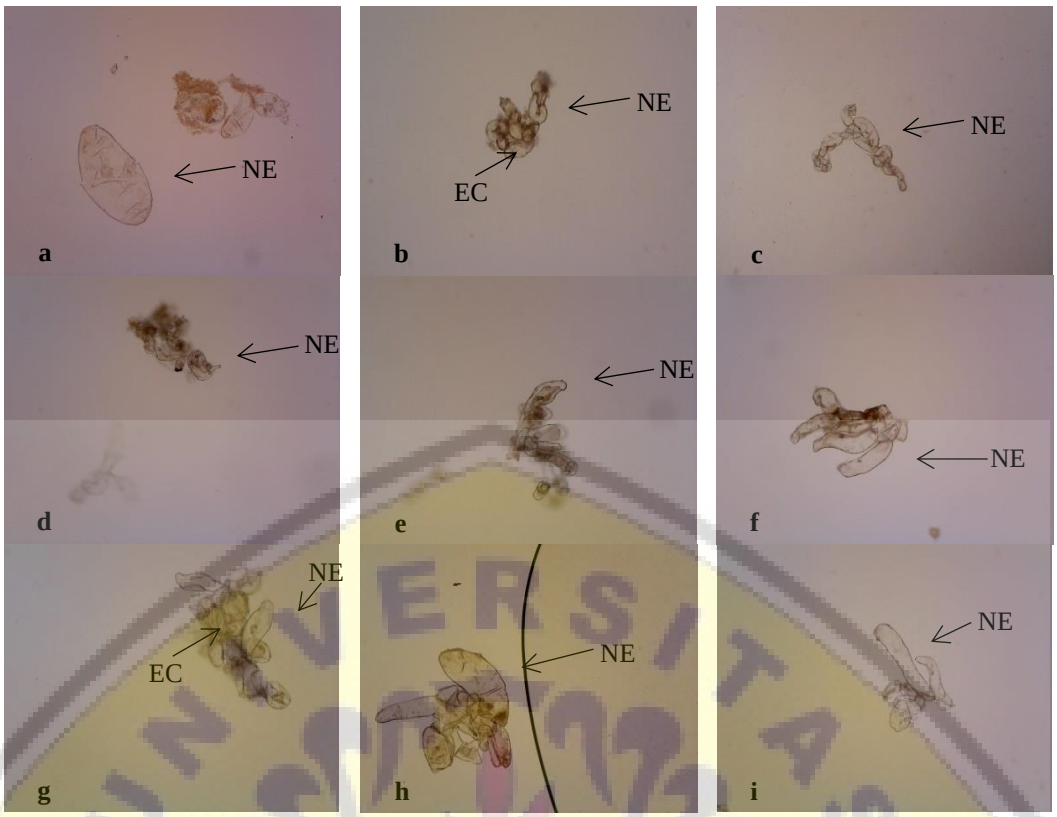

Figure 9. Callus cell obtained from 3 weeks shaking. (Magnified 4-10x) (EC: Embryogenic cell, NE: Non-Embryogenic cell). (a) pH 5.5 + sucrose 3% (A1B1). (b) pH 5.5 + sucrose 4% (A1B2). (c) pH 55 + sucrose 5% (A1B3). (d) pH 6.0 + sucrose 3% (A2B1). (e) pH 6.0 + sucrose 4% (A2B2). (f) pH 6.0 + sucrose 5% (A2B3). (g) pH 6.5 + sucrose 3% (A3B1). (h) pH 6.5 + sucrose 4% (A3B2). (i) pH 6.5 + sucrose 5% (A3B3).

The induced embryogenic callus was transferred to solid proliferation media for propagation and maturation then placed in liquid proliferation media. The callus used was embryogenic callus (Figure 3). Proliferation in liquid media was carried out for 3 weeks and shaken using a shaker at 100 rpm (Alfian *et al*, 2019). Callus was placed in dispersed media to separate the callus crumbs by shaking. A week-old callus on liquid media remained white or yellowish-white color, then started to turn brown at the 2nd and 3rd week (Figure 5, 6, and 7). Browning can be caused by the presence of phenolic compounds, stress due to cutting, or the effect of shaking (Nofrianinda *et al*., 2017). In liquid media, phenolic compounds are able to dissolve which resulting in the changes of media color into brown. The presence of browning in callus can inhibit growth and even cause cell death. Therefore, 300 mg L^{-1} of PVP was added to liquid media to prevent browning. According to Ledo *et al* (2018), the addition of 300 mg L^{-1} PVP reduces browning in liquid cultures. PVP absorbs phenol through hydrogen bonds to reduce oxidation and peroxidase content (Hutami, 2016). The main purpose of using liquid media in this study was to proliferate single cells from embryogenic callus cells. This research is part of a series of studies to optimize single cell propagation of sugarcane using liquid media. Embryogenic cells have a higher viability, which can enable single-cell-scale mass propagation in sugarcane. Ningtiyas *et al*., 2016 stated that embryogenic tissue has the potential to support the development of explant become a complete plant, while somatic embryogenesis stage which scutellar stage has developed into bipolar tissues with complete structure that had both shoot and root meristem.

The application pH 6.5 + 3% sucrose (A3B1) was the best treatment which enabled the callus to develop from globular to scutellar (Figure 5 and Figure 6), even there were no significantly different for number of selected callus among treatments in the 1st, 2nd, and 3rd week. The color of the callus from A3B1 treatment observed every week was not easily changed to brown. The callus was well dispersed then developed, and the callus cells remained embryogenic after 3 weeks at the end of the shaking period. While application pH 6.0 + sucrose 5% (A2B3) resulted in the lowest number of selected callus in the 1st week and easily turned brown.

Sucrose application of 3% was the best concentration compared to other concentrations for callus proliferation in liquid media. This is in accordance with Jamil *et al.* (2017) that 30 g L^{-1} sucrose was the best concentration for the induction of sugarcane callus. If sucrose content in the media is too high, the development of somatic embryos will be inhibited due to plasmolysis which cell fluid comes out by osmosis. The 4,5% sucrose in

sorghum caused explant darkened and secreted phenolic compound in shorgum (Dreger *et al.*, 2019). The sucrose can increase the rate of photosynthesis and embryo formation leads to the optimum absorption of water and nutrients in media. Sucrose in the media will be hydrolyzed for callus respiration and stimulate the growth of some tissues. During respiration, sucrose is converted into energy in the form of ATP (Samudera *et al*., 2019).

The pH of media also affected nutrient absorption during proliferation in liquid media. The pH of sterilized media decreased by 0.58 to 1 of acidity compared to the initial pH. A very low pH is not optimal for callus growth because nutrients in the growing media cannot be absorbed properly and inhibit cell growth. While a very high pH of media can increase polyphenol enzyme that causes the color of callus easily change to brown (Tarampak *et al*., 2019). According Xi *and* Yang (2017), during proliferation, there was no significant difference in growth of roots and shoots of apple during induction at pH 6.0-8.0, there was only a slight decrease in the number of shoots proliferation at pH >7.5. There was no significant different of number of selected callus among all of the pH treatments.

The hydrolysis of sucrose in cells affected by the temperature and pH of media, a lower pH at a certain temperature can enhance the sucrose hydrolysis. The commonly used pH of media is slightly acidic ranges from 5.5 to 5.8 then the media sterilized using an autoclave to provide the appropriate temperature for sucrose catalysis. The pH adjustment affecting the biological activity of cells. In pine trees, the optimal pH for sucrose synthesis is 7.7 and 6.7 depending on the plant variety (Chen *et al*., 2015). According to Widiastoety *et al* (2005), the commonly used pH for tissue culture media is 5.0-6.5. The pH value affects the absorption of components or nutrients in the media for cell biochemical reactions which affecting plant differentiation and morphogenesis.

Callus dispersed in a liquid medium took more than three weeks to form larger clumps. These larger clumps play an important role in reducing the occurrence of browning in the callus to be regenerated. At the clumps callus size that is too small, the phenolic stress that may occur will be higher because the cell clusters are too small to survive. Liang et al., 2018 stated that there are two main mechanisms for browning in plant tissue culture, which is enzymatic browning and stress browning caused by explant wound or culture conditions. Modification of sucrose and pH in this research are purposed to reduce browning in culture conditions, focused on callus proliferation stage.

CONCLUSIONS

Based on the obtained data, it can be concluded that the best combination treatment of pH and sucrose for sugarcane callus proliferation in liquid culture media was pH 6.5 + sucrose 3% (A3B1) with the highest number of calluses and the color did not easily turn to brown. Higher concentration of sucrose caused callus darkened and lose the ability to proliferate. More studies are needed to regenerate the dispersed callus that has been proliferated in liquid culture media.

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