

Determination of the optimum initial callus weight for the efficient propagation of sugarcane in temporary immersion bioreactor

Penentuan bobot awal kalus yang optimum untuk propagasi tanaman tebu yang efisien pada bioreaktor perendaman sesaat

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Received 19 Aug 2022 / Accepted 11 Oct 2022

Abstrak

Bioreaktor perendaman sesaat (BPS) telah digunakan secara luas untuk propagasi skala massal berbagai tanaman penting, termasuk tanaman tebu. BPS menyediakan sistem kultur semi-otomatis dan kondisi optimal bagi pertumbuhan tanaman. Beberapa faktor menentukan pertumbuhan tanaman pada BPS, salah satunya densitas dari eksplan. Oleh karena itu, penelitian dilakukan untuk menentukan bobot awal yang optimal untuk kalus tebu yang dikulturkan pada BPS, serta mengevaluasi pengaruh perbedaan bobot awal kalus tersebut terhadap proliferasi dan regenerasi kalus tebu. Kalus tebu diinduksi dari daun muda yang masih menggulung dari empat varietas tebu unggul Indonesia. Bobot awal kalus yang dikultur ke dalam bejana BPS yaitu 0,05 g; 0,1 g; 0,2 g; 0,5 g; dan 1,0 g untuk setiap bejana. Kalus kemudian melalui tahap proliferasi pada BPS sebanyak tiga siklus, kemudian kalus diregenerasi pada BPS dengan perlakuan auksin dan sitokinin. Hasil penelitian menunjukkan bahwa 0,2 g merupakan bobot awal kalus yang efisien untuk proliferasi kalus tebu pada BPS, dimana eksponensial multiplikasi kalus tercapai pada bobot awal tersebut, yaitu untuk masing-masing varietas 130,3 kali (PSKA 942), 136,8 kali (PS 094), 21,3 (PS 881), dan 12,9 kali (PS 091) setelah 12 minggu. Densitas kalus pada BPS berkorelasi negatif dengan karakteristik fisikokimia medium. Hal ini menggambarkan variasi intensitas pertumbuhan dan metabolisme kalus dengan adanya perbedaan densitas pada BPS. Penggunaan BAP 0,2 mg L⁻¹ bersama kinetin 0,2 mg L⁻¹ paling sesuai untuk memacu regenerasi kalus tebu dengan menghasilkan jumlah tunas terbanyak dalam waktu relatif lebih cepat (1 – 2 minggu lebih cepat) dibandingkan perlakuan lainnya dan dengan tingkat kejadian pencoklatan yang rendah.

[Kata kunci: kultur *in vitro*, kultur cair, proliferasi]

Abstract

Temporary immersion bioreactor (TIB) has been utilized for the mass-scale propagation of many important plants, including sugarcane. TIB facilitates a semiautomated culture system and provides optimal conditions for plant growth. Several factors determine plant growth in the TIB, such as explant density. Therefore, an experiment was carried out to determine the optimal initial weight of sugarcane calli and to evaluate its effect on the proliferation and regeneration in TIB. Sugarcane calli were induced from spindle leaves isolated from four Indonesian prime sugarcane varieties. The initial weights of the calli cultured in the TIB flasks were 0.05 g, 0.1 g, 0.2 g, 0.5 g and 1.0 g per flask. The calli were proliferated through three cycles in TIB, and subsequently regenerated in TIB with auxin and cytokinin treatments. The results of the experiments showed that 0.2 g was the most efficient initial weight for sugarcane callus proliferation in the TIB, resulting in an exponential multiplication rate of 130.3-fold (PSKA 942), 136.8-fold (PS 094), 21.3-fold (PS 881), and 12.9-fold (PS 091) within 12 weeks. In the TIB, callus density showed a negative correlation with the physicochemical properties of the medium, demonstrating various growth intensities or metabolic activities of calli at different densities in the TIB. The use of 0.2 mg L⁻¹ BAP along with 0.2 mg L⁻¹ kinetin was suitable for promoting the regeneration of sugarcane calli and producing the highest number of shoots in a relatively short amount of time (1 – 2 weeks faster) with low incidences of browning.

[Keywords: *in vitro* culture, liquid culture, proliferation]

Introduction

Sugarcane (*Saccharum officinarum* L.) is an important commodity worldwide since sugar is a staple component in the human diet. Many countries have attempted to increase national sugar production through expansion of sugarcane area and replanting, also enhancements of crop productivity. Therefore, a large quantity of seed canes is needed to support these programs. The high demand for seed canes can be met through *in vitro* culture methods. Plant propagation through *in vitro* cultures can produce substantial amounts of uniform and disease-free seedcanes and it is independent of the growth season.

In vitro culture methods for sugarcane propagation have been successfully developed since 1964 (Nickell 1964). These propagation techniques have used shoot apical tissues (Dinesh *et al.*, 2015; Redae & Ambaye, 2018) or young spindle leaves (Minarsih *et al.*, 2013; Kaur & Kapoor, 2016) as explants. Two techniques commonly used for sugarcane propagation are organogenesis (Dinesh *et al.*, 2015; Redae & Ambaye 2018) and somatic embryogenesis (SE) (Silveira *et al.*, 2013; Kaur & Kapoor, 2016; Dewanti *et al.*, 2016). Both techniques involve several specific phases, but they mostly involve the callus phase. The advantage of initiating a callus phase is that calli have high capacities to proliferate and regenerate (Ikeuchi *et al.*, 2013). Moreover, genetic analysis has revealed that *in vitro* propagation of sugarcane through callus multiplication can produce sugarcane plantlets with stable genetics for up to nine subculture cycles (Azizi *et al.*, 2020). Therefore, a method that can produce mass-scale plantlets applying a limited number of subculture cycles is needed.

The most common propagation methods use solid culture systems in which explants are cultured into jars containing solidified media. However, this system is considered inefficient at the industrial scale. Therefore, a liquid culture system is necessary to scale up seedling production by enhancing explant multiplication (Kaur & Sandhu, 2015). Moreover, scaling up requires larger culture chambers because jars do not provide sufficient amounts of space. Therefore, bioreactors must be utilized to provide more space for explant multiplication; they also reduce the number of subculture cycles required to achieve the desired yields.

Among the available bioreactor systems for plants, the temporary immersion bioreactor (TIB) is commonly used for plant propagation purposes. TIB has semiautomated systems to immerse propagules in a liquid medium for preset durations at specified intervals (Georgiev *et al.*, 2014). The system balances nutrient and gas transfer to plant cells so that hyperhydricity, which causes growth disorders, is

prevented (De Carlo, 2021) and yield is maximized (Mordocco *et al.*, 2008). Several TIB systems are available, such as TIBS BFJX-IV (Biofunction Co. Ltd, China) (Zhang *et al.*, 2018), SETIS™ (Vervit, Belgium), RALM (RALM Industria e Comercio Itda, Brazil), PLANTIMA (A-Tech Bioscientific Co., Ltd., Taiwan), PLATFORM (Plant From AB, Swedia & TC Propagation Ltd., Ireland), and RITA® (VITROPIC, Saint-Mathieu-de-Treviers, France) (Georgiev *et al.*, 2014).

Protocols for sugarcane propagation have been developed for the TIB RITA® system (Lorenzo *et al.*, 1998; Arencibia *et al.*, 2008; Mordocco *et al.*, 2008; Yang *et al.*, 2010; Minarsih *et al.*, 2013). Some factors affecting propagation in TIB have been studied. Lorenzo *et al.* (1998) optimized media in terms of volume and hormone levels, whereas Mordocco *et al.*, (2008) and Minarsih *et al.*, (2013) examined optimal immersion durations and frequencies. However, another factor affects the growth and efficiency of plant propagation in TIB, namely, the initial density or initial weight of explants (Polzin *et al.*, 2014; Zhang *et al.*, 2018; Ekmekçigil *et al.*, 2019; Aguilar *et al.*, 2019). High density leads to competition for nutrients, causing growth inhibition, while low density is inefficient for production. Therefore, it is important to determine the optimal density of explants that can be propagated in TIB to achieve maximum growth capacity. In this study, sugarcane was propagated via callus initiation, followed by callus proliferation and regeneration in TIB. The influence of explant density on the propagation of sugarcane in TIB was evaluated by measuring the initial weight of the cultured calli and by observing callus multiplication or proliferation in the TIB.

Material and Methods

Explant preparation and sterilization

The plant materials used for the experiment consisted of sugarcane varieties that are suitable for irrigated area (PS 091 and PS 881) and rainfed area (PSKA 942 and PS 094). These varieties were released and provided by the Indonesian Sugar Research Institute, Pasuruan, East Java. Spindle leaves were isolated from 6-month-old plant and used as explants. Explants were sterilized in a laminar air flow (LAF) cabinet. Sterilization was conducted by spraying the explants with 70% ethanol followed by flaming with a burner, and these steps were repeated three times following routine protocols in laboratory.

Callus initiation

Sugarcane callus initiation was conducted using a solid culture system. A medium containing Murashige & Skoog (MS) basal minerals enriched with 30 g L⁻¹

sucrose, 3 mg L⁻¹ 2,4-dichlorophenoxy acetic acid (2,4-D), and 3 g L⁻¹ gelling agent was used for callus initiation, based on a protocol by Minarsih *et al.* (2013). The medium was adjusted to pH 5.7, sterilized using an autoclave (121 °C, 1 atm, 30 minutes), and left undisturbed overnight before used. Sterile explants from the previous step were sliced into transverse sections approximately 0.5 cm in thickness and then cultured in jars containing the callus initiation medium. The cultures were then kept in a dark room and subcultured every 4 weeks.

Callus proliferation in Temporary Immersion Bioreactor (TIB)

Callus proliferation was performed in a TIB RITA® system. Calli with initial weights of 0.05 g, 0.1 g, 0.2 g, 0.5 g, and 1.0 g per flask were cultured into TIB flasks, with six replicates. Then, a liquid medium that had the same composition as the callus initiation medium but without the gelling agent was poured into the flasks. The TIB cultures were then kept in a dark room at 25 ± 2 °C for 3 cycles (4 weeks for every cycle). At the end of every cycle, the medium in the TIB was replaced with fresh medium (same composition).

Data was collected for callus weight and the physicochemical properties of the medium, such as soluble sugar level (SSL), total dissolved solids (TDS), and electrical conductivity (EC) of the medium. The SSL of the medium was measured by using a handheld Analog Refractometer 0–90% Brix Sugar. Refractometer was calibrated with distilled water before use. Afterwards, a drop of liquid media sample was placed on the prism and the cover plate was secured. The prism end of the refractometer was then pointed towards a light source and the scale value of SSL were read. The TDS and EC of the medium were measured by using a handheld digital TDS/EC meter (EZ-1, China). The sensor of TDS/EC meter was dissolved into liquid media sample and the TDS/EC value was read on the screen.

Callus regeneration in TIB

After three proliferation cycles, regeneration was induced in the calli by replacing the medium in the TIB with a regeneration medium. The medium consisted of MS basal minerals enriched with 30 g L⁻¹ sucrose and cytokinin combinations: 6-benzylaminopurine (BAP) at 0.2, 0.5, and 1.0 mg L⁻¹ and kinetin (Kin) at 0.0 and 0.2 mg L⁻¹. A regeneration medium based on Minarsih *et al.* (2013) was used for comparison, which contained 0.2 mg L⁻¹ BAP and 2.0 mg L⁻¹ indole-3-acetic acid (IAA). Each treatment had 6 TIB flasks of replicates. In the regeneration stage, the TIB cultures were kept in a room with a 12-hour photoperiod (20 µmol m⁻² s⁻¹ PPF) at 25 ± 2 °C for 3-4 cycles (4 weeks for every cycle). At the end of each cycle, the

medium in the TIB was replaced with fresh media (same composition), and the data collection was performed for the number of regenerated shoots and percentage of browning. Moreover, observation for initiation of regeneration was performed weekly.

Data analysis

The experiments were conducted with a completely randomized design. The effectiveness of the treatments on the callus weights, callus multiplication rates, and number of shoots was determined by performing analysis of variance (ANOVA). Furthermore, Tukey's test was performed at a 95% confidence level when the results showed significant differences.

Results and Discussion

Sugarcane callus proliferation in TIB with different initial callus weights

The primary calli of sugarcane emerged from the explants after 4 weeks of culture. The percentage of callus initiation was 8.9–40.4 %. The calli were friable except for PS 881 variety, which had a soft and watery callus. The calli were then proliferated in TIB with different initial callus weights.

Fig. 1 shows the progress of callus proliferation with different initial callus weights after the first, second, and third cycles of proliferation in the TIB. Based on the Fig. 1, the initial weight of the callus cultured in the TIB affected callus yield after the second cycle and affected the final yield at the end of the third proliferation cycle. The calli of sugarcane varieties that are suitable for irrigated area (PS 881 and PS 091) had lower yields than the calli of sugarcane varieties that are suitable for rainfed area (PSKA 942 and PS 094). The data indicated that sugarcane varieties that are suitable for irrigated area had slower growth response in the *in vitro* culture. However, the proliferation patterns of all varieties were similar. Generally, callus yield showed a positive correlation with callus initial weight. The calli of PSKA 942, PS 091, and PS 881 showed exponential rates of proliferation based on their final yields with 1.0 g of initial weight. The yield of the PSKA 942, PS 091, and PS 881 callus were 86.78 g, 8.33 g, and 13.73 g respectively, whereas PS 094 showed an exponential growth of 50.88 g, starting with 0.5 g initial weight.

The different initial weights did not affect the multiplication rates of the sugarcane calli during the first proliferation cycle (Fig. 2). The initial weights affected the multiplication rate of the calli after two proliferation cycles in PSKA 942 and PS 881 varieties, and after three cycles in PS 094 variety. Based on statistical analyses, the highest callus multiplication rate of PSKA 942 was obtained with

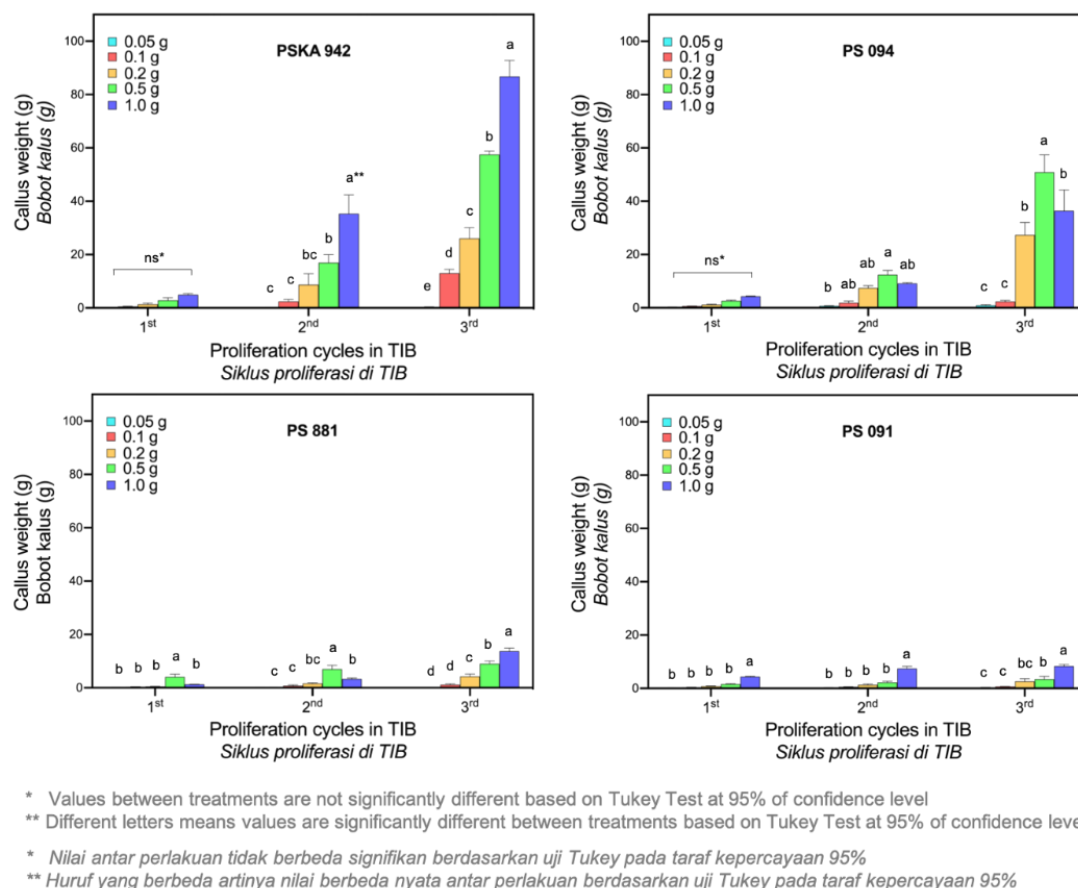


Figure 1. Proliferation of sugarcane calli in TIB with different initial callus weights
 Gambar 1. Proliferasi kalus tebu pada TIB dengan bobot awal kalus yang berbeda

initial weights of 0.2 g and 0.1 g, reaching 130.3- and 129.7-fold, respectively, at the end of the third cycle. In PS 094 variety, the highest callus multiplication rate was obtained with an initial weight of 0.2 g (136.8-fold), and in PS 881 variety, the highest callus multiplication rate was obtained with initial weights of 0.2 g and 0.5 g, reaching 21.3- and 18.1-fold, respectively, at the end of the third cycle. In contrast, the callus multiplication rate of PS 091 in TIB did not influence by the initial weights (Fig. 2).

The relative growth of sugarcane calli of different initial weights in the TIB was also reflected in the physicochemical parameters of the TIB medium. The soluble sugar levels (SSLs) of the proliferation media were significantly affected by the different initial weights of calli (Fig. 3). Lower initial weights resulted in higher SSLs in the media. The initial SSL of the medium was 4. After the first proliferation cycle, the SSL in the TIB media with the calli of 0.05 g initial weight was unchanged in PSKA 942, PS 881, and PS 091 varieties, while there was a slight reduction in the SSL for PS 094 variety (3.6). Overall, the SSL for the calli of 0.05 g initial weight in the TIB was not less than 3.3 even after the third cycle. In contrast, the TIB

that received initial callus weights of 0.5 g and 1.0 g showed significant reductions in SSLs after the second cycle in all varieties. Moreover, the SSL was nearly zero for PSKA 942 and PS 094 varieties after the third cycle.

The initial weight of the callus cultured in the TIB also influenced the total dissolved solids (TDS) concentrations of the media (Fig. 4). The initial TDS in the medium was approximately 3,500 ppm. The initial callus weight of PSKA 942 influenced TDS concentrations after the second proliferation cycle, especially for 0.5 g and 1.0 g initial callus weights. After the third cycle, the TDS concentrations decreased to 1,667 ppm and 1,510 ppm, respectively. In PS 094 variety, only the 0.5 g and 1.0 g initial callus weights influenced the TDS concentrations. After the third cycle, TDS concentrations decreased to 1,586 ppm and 1,841 ppm, respectively. However, in two other varieties that are PS 881 and PS 091, TDS concentrations were influenced by the callus initial weight after the first proliferation cycle. The TDS concentrations after the first and second cycles were approximately 3,000 ppm, and after the third cycle was approximately 2,500 ppm.

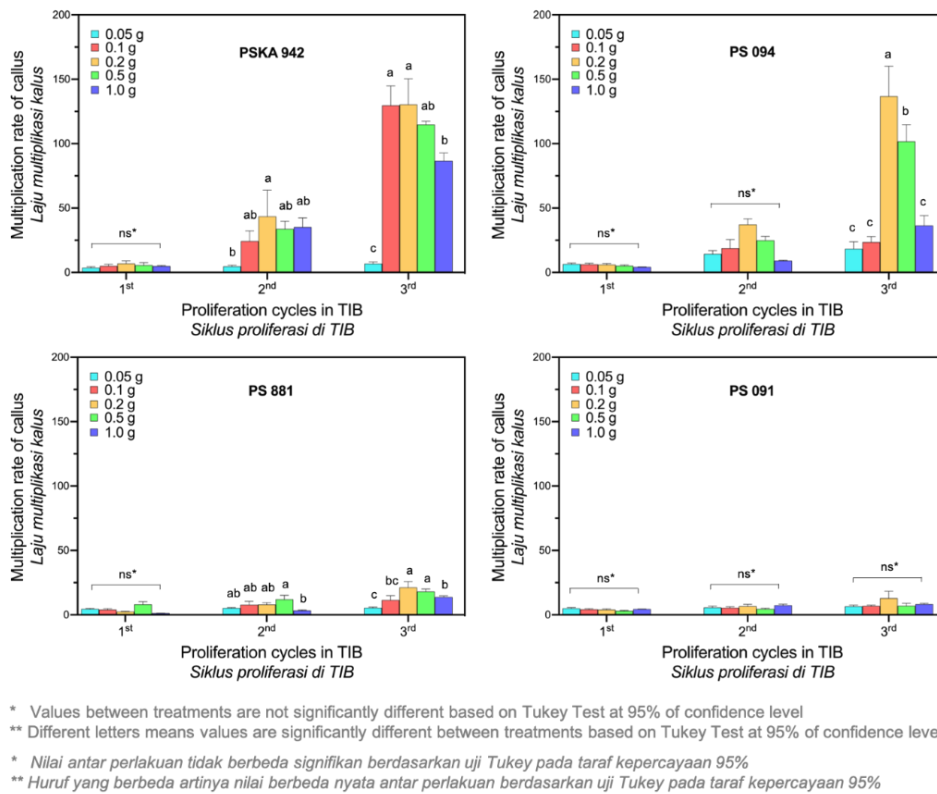


Figure 2. Multiplication rate of sugarcane calli in TIB with different initial callus weights
 Gambar 2. Laju multiplikasi kalus tebu pada TIB dengan bobot awal kalus yang berbeda

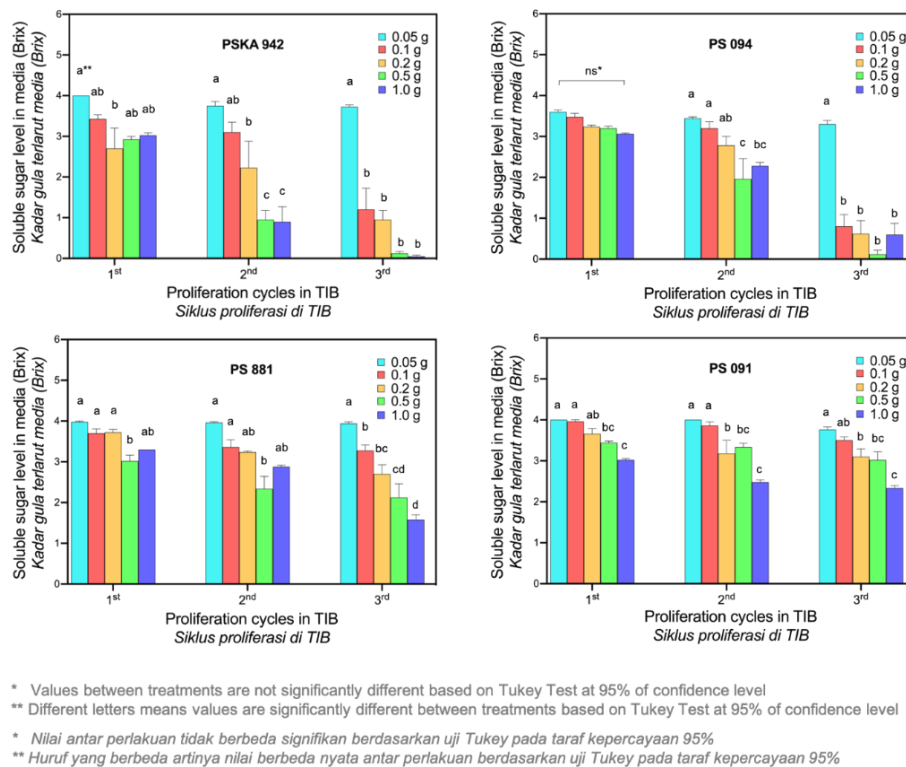


Figure 3. Soluble sugar level of the media for sugarcane callus proliferation in TIB with different initial callus weights
 Gambar 3. Kadar gula terlarut pada media proliferasi kalus di TIB dengan bobot awal kalus yang berbeda

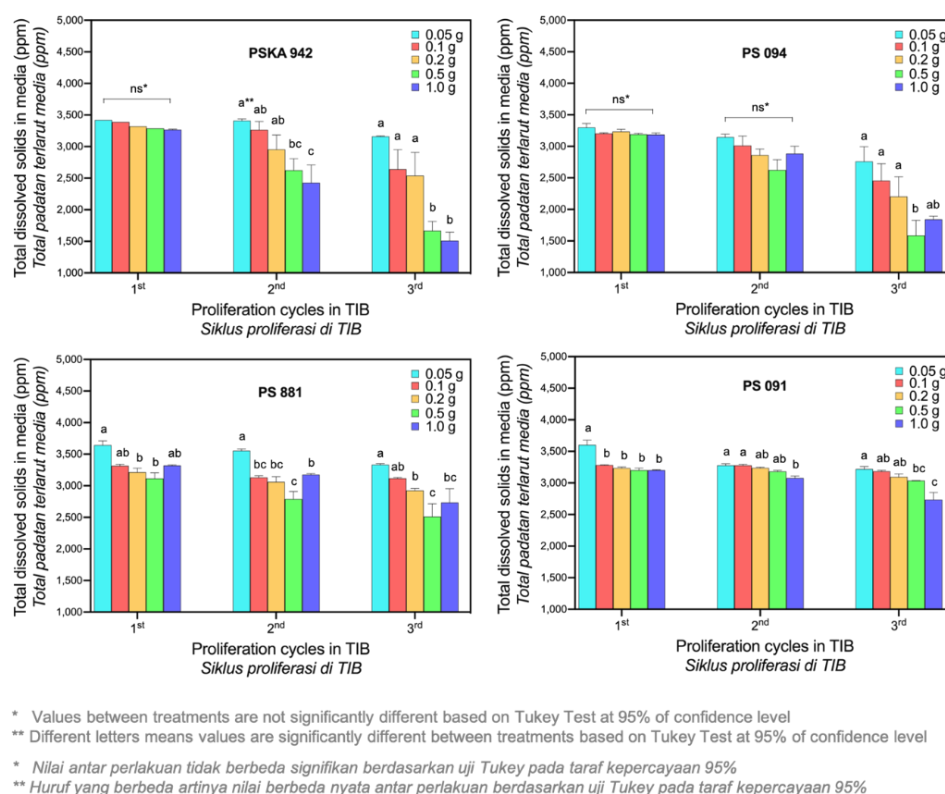


Figure 4. Total dissolved solids of the media for sugarcane callus proliferation in TIB with different initial callus weights
 Gambar 4. Total padatan terlarut pada media proliferasi kalus di TIB dengan bobot awal kalus yang berbeda

Fig. 5 shows the measurement results for the electrical conductivity (EC) of the medium in the TIB with different initial callus weights. The EC trend was similar to that of TDS. The initial EC was approximately $7,000 \mu\text{S cm}^{-1}$. The initial callus weight of PSKA 942 variety influenced EC after the second proliferation cycle, especially for 0.5 g and 1.0 g initial callus weight. After the second cycle, EC decreased to $5,250 \mu\text{S cm}^{-1}$ and $4,634 \mu\text{S cm}^{-1}$, respectively, and after the third cycle, it was $3,335 \mu\text{S cm}^{-1}$ and $2,508 \mu\text{S cm}^{-1}$, respectively. Similarly, only the 0.5 g and 1.0 g initial callus weights of PS 094 influenced EC of the medium in the TIB. After the third cycle, EC decreased to $3,173 \mu\text{S cm}^{-1}$ and $3,309 \mu\text{S cm}^{-1}$, respectively. In contrast, for PS 881 and PS 091 varieties, EC values were influenced by the initial callus weight after the first proliferation cycle. The lowest EC in PS 881 variety was $5,034 \mu\text{S cm}^{-1}$ with the 0.5 g initial callus weight after the third cycle. The lowest EC in PS 091 variety was $5,470 \mu\text{S cm}^{-1}$ with the 1.0 g initial callus weight after the third cycle.

It is generally assumed that a high initial callus weight results in a high final callus yield to some extent. The results in this study also agreed with that hypothesis (Fig. 1). However, a large population can cause high metabolic activity, as indicated by the high depletion of sugar and dissolved solids in the media (Fig. 3 & Fig. 4). For example, in PSKA 942 variety,

which had the highest growth among the varieties, the 0.5 g and 1.0 g initial callus weight treatments resulted in the highest final callus yields at the end of the second cycle (16.88 g and 35.27 g , respectively) and third cycle (57.43 g and 86.77 g , respectively) (Fig. 1). Accordingly, in those treatments, the SSLs at the end of the second and third cycles were nearly zero (Fig. 3), and the TDS was below $2,500 \text{ ppm}$ (Fig. 4). The physicochemical parameters of the media represented nutrients used for explant metabolism in the TIB (Uma et al., 2021). Sufficient quantities of nutrients must be available for the optimal growth of all explant cells in the TIB.

Therefore, the efficiency of callus proliferation in the TIB is reflected in the multiplication rate instead of the final yield. Fig. 2 demonstrates that most sugarcane varieties (PSKA 942, PS 094, PS 881) reached an exponential callus multiplication rate with the 0.2 g initial callus weight treatment at the 3rd cycle of proliferation. With these treatments, the calli of PSKA 942 and PS 094 varieties reached approximately 130-fold their initial weight, while PS 881 variety reached 21-fold and PS 091 variety reached 12-fold by the end of the third proliferation cycle. This indicates that nutrients in the media and the space in the TIB were sufficient for the optimal growth of 0.2 g initial callus weight. A high initial callus weight resulted in overcrowding (high density)

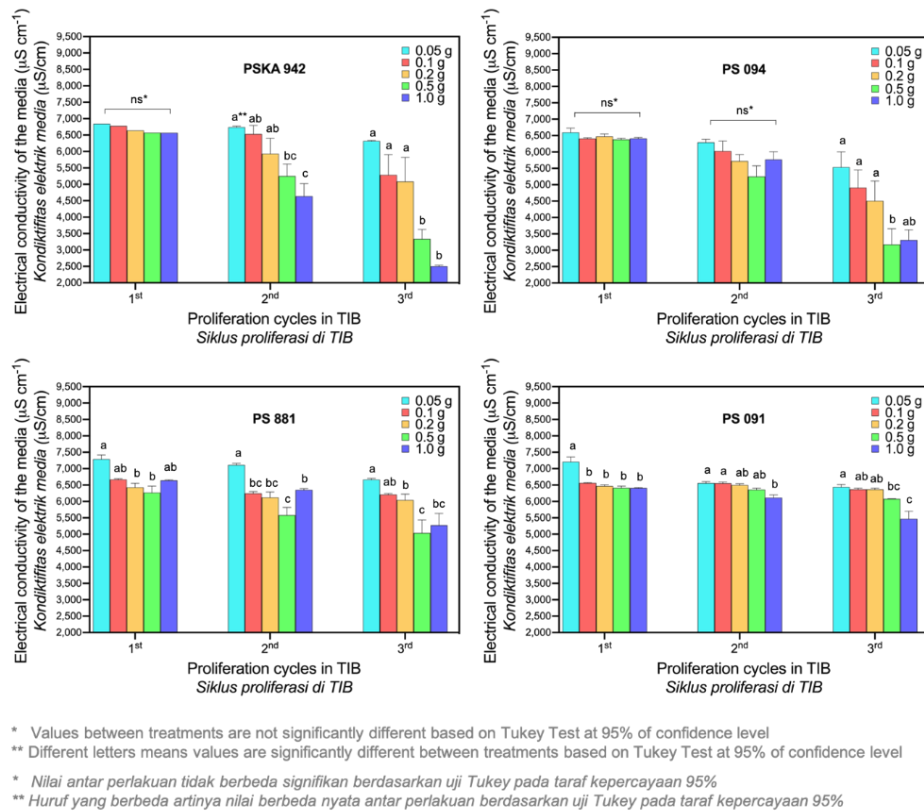


Figure 5. Electrical conductivity of the media for sugarcane callus proliferation in TIB with different initial callus weights
 Gambar 5. Konduktifitas elektrik pada media proliferasi kalus di TIB dengan bobot awal kalus yang berbeda

in the final proliferation stages, which might cause nutrient and gas deficiencies. Some cells in the TIB culture barely received nutrients; therefore, they could not fully replicated, and some even died.

Other researchers have also reported limitations in explant multiplication rates caused by excessive densities in TIB (San José *et al.*, 2020; Bello-Bello *et al.*, 2021). In contrast, low initial callus weights can result in inefficient propagation due to excessive use of nutrients and space. The callus, as an explant cultured in TIB, requires nutrients and other microclimatic support for its growth and development. The availability of nutrients, gases, and space for explant in TIB is restricted by explant density (Bello-Bello *et al.*, 2021; Uma *et al.*, 2021). Similar to cell cultures of microalgae, microbes, protoplasts, or other plant cells, the growth of calli that are grown in bioreactors is directly related to their initial density (Cheng *et al.*, 2018; Li *et al.*, 2021). Based on the above results, the initial density of 0.2 g is suggested for sugarcane callus proliferation in TIB.

Sugarcane callus regeneration by cytokinin treatment in TIB

After proliferation, the calli were regenerated in the TIB. Table 1 and Fig. 6 show that regeneration of the sugarcane calli started 6 weeks after cultured in the regeneration medium. Only PSKA 942 and PS 094

varieties regenerated shoots, while other varieties stayed in the callus phase until third cycles in the regeneration medium. The response of explant cells in the *in vitro* culture starts at the initiation, often affected its response in the next stages of cultures. At the callus initiation stages, calli derived from PSKA 942 and PS 094 were friable with relatively small and dense cells, while calli derived from PS 091 and PS 881 were clumped and watery. According to Sane *et al.* (2006) friable calli tend to grow faster and has better regeneration capacity than clumped calli. Consequently, in this research, for the next stages of culture such as callus proliferation and regeneration, PSKA 942 and PS 094 varieties have higher proliferation and regeneration rates than PS 091 and PS 881 varieties. Thereupon, PS 091 and PS 881 varieties might need further treatments optimization for better response in the *in vitro* culture.

According to Table 1, generally, the use of cytokinin in combination (BAP + Kin) allowed the sugarcane shoots to grow faster than the use of BAP alone. The highest number of shoots was obtained following the cytokinin combination treatments : BAP 0.2 mg L⁻¹ + Kin 0.2 mg L⁻¹ and BAP 1.0 mg L⁻¹ + Kin 0.2 mg L⁻¹. The treatments on average produced 91 and 112 shoots per TIB flask, as observed at 8 weeks of culture. Treatment with BAP without Kin

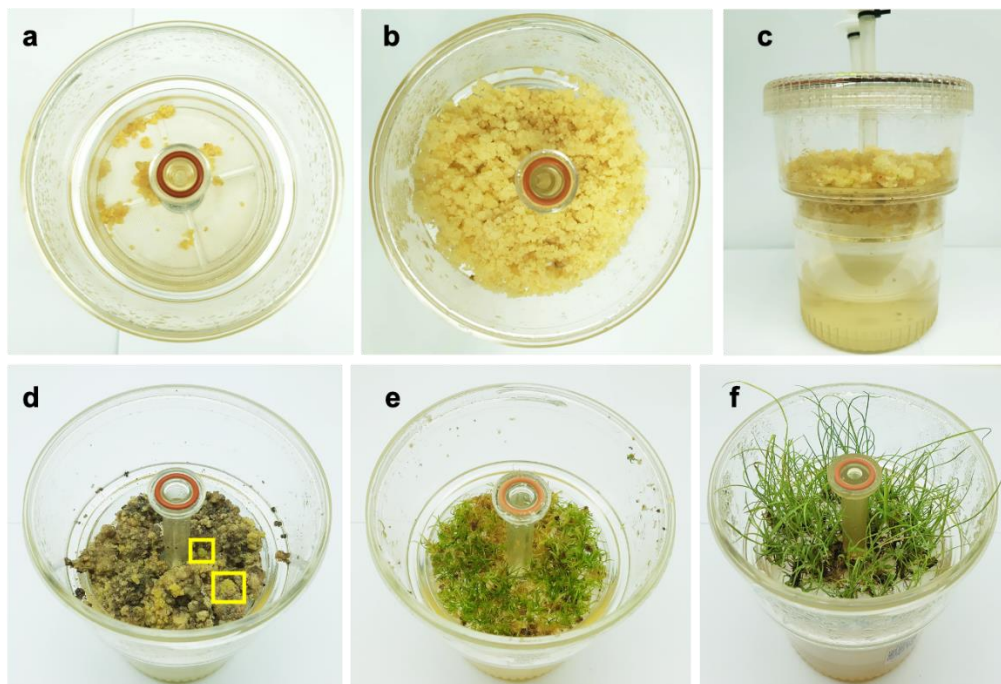


Figure 6. Sugarcane callus proliferation in TIB: 0.2 g of cultured callus (a) and after three cycles of proliferation (b,c). Sugarcane callus regeneration in TIB: initial sugarcane shoots (yellow box) and incidences of browning (d), shoots regenerated into plantlets (e,f). Diameter of flask(s): 12 cm

Gambar 6. Proliferasi kalus tebu pada TIB: 0,2 gram kalus yang dikultur (a) dan setelah tiga kali siklus proliferasi (b,c). Regenerasi kalus tebu di TIB: tunas awal tebu (kotak kuning) dan kejadian pencoklatan (d), tunas-tunas yang tumbuh menjadi planlet (e,f). Diameter bejana: 12 cm

produced shoots after 8 weeks of culture. The use of BAP alone was the best at inducing callus regeneration at 0.5 mg L^{-1} , resulting in an average of 54.7 shoots per TIB flask. The lowest callus regeneration occurred following the treatment of 1.0 mg L^{-1} BAP, resulting in an average of 6 shoots per TIB flask. In contrast, the reference medium (Minarsih *et al.*, 2013), which contained a combination of BAP and IAA, produced fewer shoots. This result agreed with studies by Suprasanna *et al.*, (2005) and Silveira *et al.*, (2013), in which better *in vitro* regeneration of shoots was induced by the reduction or by the absence of auxins in the medium. Auxins, such as IAA, mainly affect root initiation (Yoshida *et al.*, 2013; Mustafa & Khan, 2015; Gómez-Kosky *et al.*, 2020), while cytokinins, such as BAP and Kin, mainly promote shoot regeneration (Hill & Schaller, 2013; Motte *et al.*, 2014). Furthermore, Kaur & Kapoor (2016) reported on the use of BAP and Kin for inducing sugarcane callus regeneration, achieving an 81–94% regeneration rate.

Browning rates were high in most treatments (Table 1). Observations (Fig. 6d) showed that treatment with BAP 1.0 mg L^{-1} + Kin 0.2 mg L^{-1} caused the highest explant browning at 70%, while the reference medium from Minarsih *et al.*, (2013)

that used a combination of BAP and IAA resulted in the lowest incidences of browning, at 15%. Browning occurs due to the oxidation of secondary metabolites, such as polyphenols, in response to stress in plant cells (Jones & Saxena, 2013; Chuanjun *et al.*, 2015). The oxidation reaction is catalyzed by polyphenol oxidase (PPO), forming the brown pigment melanin, which is responsible for browning (Chuanjun *et al.*, 2015). In most cases, browning inhibits callus regeneration in many plant species (Duan *et al.*, 2018; Zhang *et al.*, 2020; Rahman *et al.*, 2021). In this experiment, the highest browning rate occurred in the treatment that produced the highest number of shoots. The treatment contained the highest concentration of cytokinin among the treatments, which may have caused stress in the plant cells. In contrast, treatment with 0.2 mg L^{-1} BAP + 0.2 mg L^{-1} Kin, which contained a lower concentration of BAP, also produced a high number of shoots (91.8) in a relatively short period of time (started after 7 weeks of culture) but reduced the rate of browning (30%) (Table 1). Therefore, this treatment could be used as an alternative for inducing sugarcane callus regeneration in TIB with low rates of browning.

Table 1. Regeneration and browning rate of sugarcane calli in TIB with cytokinin treatments

Tabel 1. Regenerasi dan tingkat pencoklatan kalus tebu pada TIB dengan perlakuan sitokinin

Treatments Perlakuan	Initial generated shoots Waktu awal terbentuknya tunas	Number of shoots Jumlah tunas	Explant browning rate Tingkat pencoklatan eksplan
BAP 0.2 + Kin 0.0	8 weeks	17.8 cd*	45%
BAP 0.5 + Kin 0.0	8 weeks	54.7 bc	35%
BAP 1.0 + Kin 0.0	8 weeks	6.0 d	52%
BAP 0.2 + Kin 0.2	7 weeks	91.8 ab	30%
BAP 0.5 + Kin 0.2	6 weeks	15.5 cd	60%
BAP 1.0 + Kin 0.2	6 weeks	112.2 a	70%
Minarsih <i>et al.</i> (2012)	6 weeks	16.3 cd	15%

*Different letters mean the values are significantly different between treatments based on Tukey's test at the 95% confidence level

*Huruf berbeda menunjukkan nilai yang berbeda signifikan antar perlakuan berdasarkan uji Tukey pada taraf kepercayaan 95%

Conclusion

The initial weight or density of callus influenced the efficiency and effectiveness of its proliferation in TIB. The initial weight of 0.2 – 0.5 g was the most efficient for sugarcane callus proliferation in TIB, in which the exponential multiplication rate was reached. Furthermore, the use of 0.2 mg L-1 BAP along with 0.2 mg L-1 kinetin was suitable for promoting the regeneration of sugarcane calli, producing the highest number of shoots in a relatively short amount of time with low incidences of browning.

Acknowledgements

This research was financially supported by the Indonesian Endowment Fund for Education (LPDP-RI) through the Riset Inovatif Produktif (RISPRO) Invitasi 2020 program.

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