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Full Length Article

Development of Synthetic Seeds Derived from Coleoptile of Sugarcane (*Saccharum officinarum*) through Somatic Embryogenesis

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Abstract

Developing synthetic seed by encapsulation technique have been considered to support seedling production for facilitating sugarcane conservation and distribution in large and wide scale. Synthetic seed technology has high potential application for supporting sugarcane seedlings production. This study investigated a comprehensively synthetic seeds production derived from somatic embryo at coleoptile stage in sugarcane including callus production, molecular analysis of somatic embryogenesis genes, up to synthetic seeds germination and regeneration. Results showed that 4 mg L^{-1} of 2, 4-D was better for inducing embryogenic callus production whereas lower 2,4-D combined with L-proline and casein hydrolysate was required to increase cell growth and the number of formed coleoptiles. The SERK, BBM, and LEC were identified in embryogenic and non-embryogenic callus formed during induction stage. Sugarcane coleoptiles were optimum to be encapsulated in 3% of sodium alginate concentration. After 48 days incubation in regeneration media, plantlet in 3% of sodium alginate resulted in better growth and development particularly in number of plantlet formation and height. © 2021 Friends Science Publishers

Keywords: Embryogenic callus; Encapsulation; RT-PCR; Sodium alginate; 2, 4-D

Introduction

Micropropagation technology or plant propagation through tissue culture has developed rapidly, especially for synthetic seed production. Synthetic seeds are considered as one of modern techniques for providing viable seed through encapsulation of somatic embryo or other meristematic part which can be regenerated into a plant. Advance propagation through synthetic seeds is important alternative to scale up seedlings especially for elite genotype of sugarcane. Synthetic seeds of sugarcane are able to be stored within a certain period (Ravi and Anand 2012). Damage resistant in synthetic seed is potential to be utilized for propagation and conservation (Sharma *et al.* 2013) and expand seedling shipping distribution (Rihan *et al.* 2017). The application of synthetic seed technology has promising prospects to support sugarcane seedlings production, including for conservation and preservation of sugarcane genotype.

Somatic embryos, zygotic embryos, buds, apical buds, axillary buds and callus from tissue culture process could become a source of explant for synthetic seeds production. Somatic embryo propagation through somatic embryogenesis is widely used for production of synthetic seeds (Helal 2011). This method forming the propagules rapidly in relative short time (Raza *et al.* 2012) which increases the success of its transformation (Heringer *et al.* 2015) and production of virus-free plant especially in sugarcane (Dewanti *et al.* 2016a). There are several factors influencing the occurrence of somatic embryo for synthetic seed encapsulation including plant growth regulators (Dewanti *et al.* 2016b), explant types, and plant genotypes (Damayanti *et al.* 2018). Application of exogenous auxin (Tahir *et al.* 2011; Sardar *et al.* 2016) and amino acid source also effect the production of somatic embryo. Amino acids addition stimulates the communication between cells and tissues in multicellular organs. Nitrogen derived from amino acids is rapidly assimilated during metabolism, which is used for protein synthesis in cell.

Suitable application of plant growth regulator and amino acid trigger the formation of somatic embryogenesis stages in sugarcane. Somatic embryo is initiated from restructured somatic cell to develop embryogenic cells (Yang and Zhang 2010). These cells experience several morphological and biochemical pathways through

induction, proliferation, and regeneration stages. Previous studies identified several genes that play important role in somatic embryogenesis process such as *Somatic Embryogenesis Receptor Kinase* (*SERK*) (Ahmadi *et al.* 2016; Porras-Murillo *et al.* 2018), *Baby Boom* (*BBM*) (Florez *et al.* 2015; Horstman *et al.* 2017), *Leafy Cotyledon* (*LEC*) (Kumar and Van Staden 2017) and *Wuschel* (*WUS*) (Bouchabké-Coussa *et al.* 2013). However, the expression of these genes during embryogenic phase in sugarcane are still undisclosed.

Induction stage requires an optimal concentration of auxin to induce competent callus. Proliferation stage is the crucial stage for producing high quality of somatic embryo. It required involvement of auxin just as 2, 4-D and combination of amino acids such as casein hydrolysate and L-Proline. Under optimum media composition, embryogenic callus develops into pro embryogenic mass (PEM), globular, scutellar, and coleoptile. Coleoptile was reported to be a suitable stage for synthetic seed germination (Inpuay and Te-Chato 2012; Ningtiyas *et al.* 2016).

Encapsulation of sugarcane coleoptile using proper gelling agent is required to preserve somatic embryo. Gelling agent using sodium alginate and calcium chloride acts as artificial endosperm for somatic embryo growth (Rihan *et al.* 2017). Proper concentration of sodium alginate is very important to be optimized to create suitable coating for somatic embryo. This coating characteristic affects the germination and regeneration level of plantlet. In Indonesia, production of synthetic seeds particularly in sugarcane is limited. This study investigated a comprehensive study of the synthetic seeds production derived from somatic embryo at coleoptile stage in sugarcane including callus production, molecular analysis of somatic embryogenesis genes, until synthetic seeds germination and regeneration.

Materials and Methods

Experimental Material

Experiments were performed in the Center for Development of Advanced Science, University of Jember, Indonesia during February 2018 – December 2019. A healthy 6 months old sugarcane plants variety NXI 1-3 were used as experimental material. The outer part of leaves tip were sterilized using alcohol 70% for 5 min and discarded until 4-5 layers of green leaves tip. The inner part of leaves tip cleaved to collect spindle leaf and sliced to 0.5 cm thickness as explants.

Callus Induction

Explants are placed in callus induction media for 6 weeks, at a temperature of 24°C under dark condition to induce embryogenic callus. Callus induction media consisted of MS (Murashige dan Skoog) supplemented with 30 g L^{-1} sucrose + 2.5 g L^{-1} phytagel + 300 mg L^{-1} Casein Hydrolisate. The media of callus induction (CI) comprised of: (CI1) 0 mg L^{-1} 2, 4-D; (CI2) 3 mg L^{-1} 2, 4-D; (CI3) 4 mg L^{-1} 2, 4-D. Formation and percentage of callus were observed by macroscopic and microscopic observations using a stereo microscope (*Leica EZ4HD*).

RT-PCR Analysis

Embryogenic and non-embryogenic callus were collected from induction phase for RNA isolation analysis. RNA isolation was conducted using *iScript cDNA Synthesis Kit* (Bio-Rad). Isolated mRNA from callus converted to cDNA through reverse transcription (RT) and amplified by PCR (Biorad t 100) using specific primer (SERK, WUS, BBM, and LEC gene). Electrophoresis using agarose gel 1% conducted for PCR product visualization.

Proliferation of Somatic Embryo

Pro Embryo Mass (PEM) from callus induction stage was transferred to proliferation media to induce somatic embryogenesis formation. PEM incubated in proliferation media for 6 weeks (4 weeks darkness, 2 weeks light) under 1600 lux at 24ºC. Proliferation media contained MS media, 30 g L^{-1} sucrose + 2.5 g L^{-1} phytagel, and 2 mg L^{-1} 2,4-D + 500 mg L^{-1} L-Proline + 300 mg L^{-1} Casein Hydrolisate. Proliferation stage determined the development of pre-embryo, globular, scutellar and coleoptile.

Synthetic Seed Production

Preparing Embryo

In coleoptile stage, embryos were collected from proliferation stage. Coleoptiles from somatic embryo were selected uniformly (± 0.5 cm) from the explants and sown into sodium alginate solution.

Encapsulation

Encapsulation solution comprised of media MS + 1.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA and three different concentrations (3, 4 and 5%) of sodium alginate. Coleoptilar embryo was mixed with encapsulation solution, piped by pipette pasteure and put in hardening solution. Capsule (*synthetic seed*) placed in $CaCl_2.2H_2O$ 100 m*M*, soaked for 30 min and rinsed with sterile distilled water 3 times, then placed on a filter paper and air dried.

Germination and Regeneration

Synthetic seeds were grown in media germination and regeneration. Regeneration media consisted of MS, 30 g L^{-1} sucrose + 2.5 g L^{-1} phytagel, 24°C, 1600 lux light intensity, 16 h light and 8 h darkness. Plantlet observed in ±2 cm heights. Germinated synthetic seeds were observed daily.

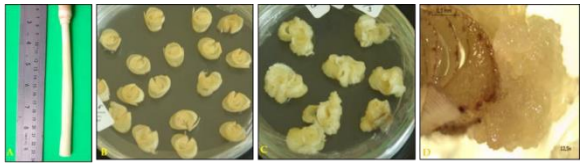

Fig. 1: Callus induction of sugarcane for synthetic seeds production: (**A**) Young leaf tip from the field for explant; (**B**) Spindle leaf section cultured on callus induction media; (**C**) Embryogenic callus on outer layer of spindle leaf; (**D**) Microscopic observation of embrogenic callus on 12.5 x magnification

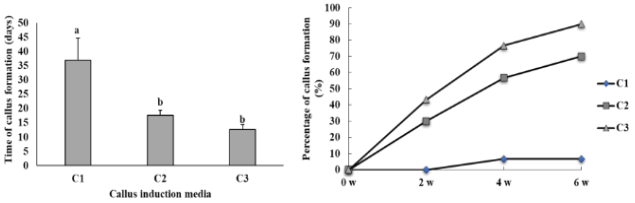

Fig. 2: Time of callus formation: (**A**) First callus formation and its percentage; (**B**) Percentage of callus formation during 6 weeks embryogenic callus induction period (C1 media: 0 mg L^{-1} 2, 4-D, C2 media: MS + 3 mg L^{-1} 2, 4-D, and C3 media: MS + 4 mg L^{-1} 2, 4-D

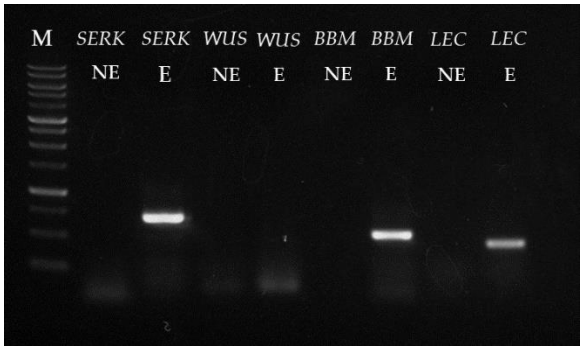

Fig. 3: Expression of *SERK*, *WUS*, *BBM*, and LEC genes on non-embryogenic (NE) and embryogenic callus (E) in sugarcane

Shoot growth of plantlet were measured after plant height reached 0.5 cm.

Results

Somatic embryogenesis was initiated from very young leaf tip of sugarcane, which named commonly as spindle leaf, as meristematic tissue to induce callus production on induction media containing 2, 4-D under dark condition (Fig. 1A and 1B). Incubation of spindle leaf on callus induction media stimulated formation of swollen tissue as callus initiation process. In consequence of cell division process, swollen tissue developed into embryogenic callus on outer layer of spindle leaf (Fig. 1C) showed smooth, white and glossy structure, and this particular spot potentially develop into next stage of somatic embryogenesis (Fig. 1D).

Addition of exogenous plant growth regulator particularly auxin to induction media also contributed on callus development. Result showed that 3 mgL^{-1} and 4 mgL^{-1} of 2, 4-D more potential for accelerating sugarcane callus initiation process than without application of 2, 4-D. Addition of 4 mgL^{-1} of 2, 4-D (C3) induced callus initiation after 12.67 days, five days faster than 3 mgL^{-1} of 2, 4-D (C2). Data showed that auxin played important role in callus induction. Without application of 2, 4-D, it required up to 37 days for callus initiation (Fig. 2A).

Application of 3 mgL^{-1} and 4 mgL^{-1} of 2,4-D also increased percentage of callus production during incubation on induction medium 70 and 90% respectively. Otherwise, MS medium without 2, 4-D delayed percentage of callus production until 6.67% (Fig. 2B). Callus induction without growth regulator intervention resulted non-embryogenic callus and less of embryogenic callus. High production of embryogenic callus stimulated higher development of somatic embryo on proliferation stage.

Identification of embryogenic and non-embryogenic callus using molecular marker required to convince the ability of callus to develop into somatic embryo. In this study, the expression of SERK, BBM and LEC genes on sugarcane embryogenic callus were discovered (Fig. 3). Interestingly, WUS gene did not identified on both types of sugarcane callus.

Composition media of proliferation also played important role to accelerate embryogenic callus production. Application of amino acid such as proline and casein hydrolysate influenced total callus weight and the number of formed coleoptiles. Presence of single casein hydrolysate produced lower total callus weight per 100 mg than combination of casein hydrolysate with proline (Fig. 4A). Based on the data, 300 mgL^{-1} of casein hydrolysate was the optimum concentration for somatic embryo development at proliferation. After combining with proline, total callus weight significantly increased approximately 45%. However, application of proline at concentration 550 mgL^{-1} tended to decline total callus weight per 100 mg about 43% (Fig. 4B).

Proper composition and concentration of media determined optimum development of somatic embryogenesis on both induction and proliferation stage. Each stage had various time initiations dependent on specific nutrient applied on media (Fig. 5). In this research, development of SE initiated from callus to Pro Embryo Mass (PEM) structure. Production of PEM structure from callus induced globular formation after 14 days incubation under optimum condition. Totally, induction phase from callus to globular structure required 42 days under dark condition.

Globular stage characterized by yellowish white to glossy of smooth clumps. After incubating under dark condition, embryo somatic transferred into light condition to promote scutellar stage. Scutellar initiation required shorter time than globular initiation. Round clumps of globular stage turned into heart shape. Some parts displayed green

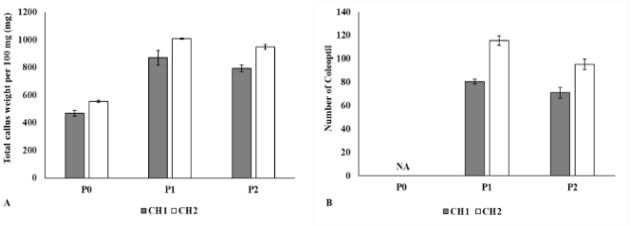

Fig. 4: Callus growth and development during proliferation (**A**) Total callus weight per 100 mg and (**B**) Number of coleoptiles under different combination of proline and casein hydrolysate concentration. (P0CH1: 0 mg L^{-1} proline and 250 mg L^{-1} casein hydrolysate; P0CH2: 0 mg L^{-1} proline and 300 mg L^{-1} casein hydrolysate; P1CH1: 500 mg L^{-1} proline and 250 mg L^{-1} casein hydrolysate; P1CH2: 500 mg L^{-1} proline and 300 mg L^{-1} casein hydrolysate; P2CH1: 550 mg L^{-1} proline and 250 mg L^{-1} casein hydrolysate; P2CH2: 550 mg L^{-1} proline and 300 mg L^{-1} casein hydrolysate). NA: not available

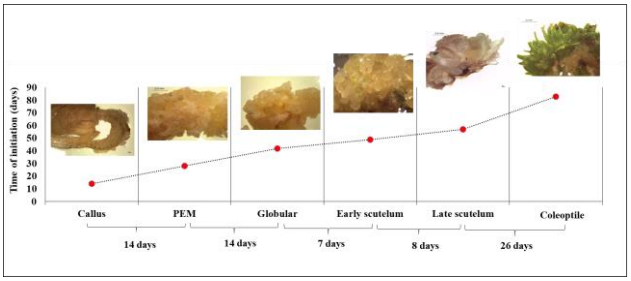

Fig. 5: Development of somatic embryogensis stages during proliferation

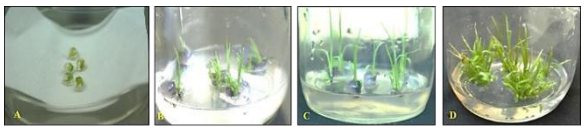

Fig. 6: Synthetic seeds germination and regeneration of plantlet: (**A**) Synthetic seeds encapsulation; (**B**) seed germination after 7 days; (**C**) Regeneration of plantlet after 14 days; and (**D**) after 48 days

spots and further developed into elongation shape. Coleoptile structure formed after 26 days, characterized by the emergence of bipolar structure green leaves. Coleoptile from embryo somatic was collected for encapsulation of synthetic seeds (Fig. 6A).

Synthetic endosperm for encapsulation used sodium alginate as nutrition storage for somatic embryos growth. Nutrient absorption of synthetic seeds was influenced by concentration of sodium alginate. Proper sodium alginate concentration enables synthetic seeds germinated after 7 days (Fig. 6B). Data showed that optimum concentration of sodium alginate for synthetic seeds encapsulation was 4%. Lower germination percentage of synthetic seeds in 5% sodium alginate concentration expected inhibit embryos growth due to hard structure of seed to be penetrated. Concentration of sodium alginate also influenced number of shoot growth after 14 days (Fig. 6C). Concentration of 3 and 4% of sodium alginate supported high number of shoot production. However, high of number of shoot production did not decide the quantity and quality of plantlet formed. After 48 days incubation in regeneration media, plantlet in 3% of sodium alginate showed better growth particularly in number of plantlet formation and its height.

Discussion

Somatic embryogenesis has several specific stages starting with the formation of pro-embryonic mas (PEM) followed by somatic embryo formation maturation, and regeneration (Fehér 2015). Somatic embryogenesis was induced from young leaf tip in sugarcane to provide optimal source for callus induction due to its totipotent character (Yasmin *et al.* 2011). Callus from meristematic tissue appeared from sliced region of spindle leaf explant and developed into embryogenic callus (Fig. 1).

Embryogenic callus were discovered and characterized by dry and yellowish-white which indicated that callus had a dense cytoplasm and high ratio of nucleus or cytoplasm (Alcantara *et al.* 2014). These characteristics showed that cell have meristematic zones for somatic embryo development. Non embryogenic callus were also identified during this study. Non embryogenic callus mostly compact, wet, transparent structure and some showed browing region (Widuri *et al.* 2016).

There are several different features of embryogenic and non-embryogenic callus based on histology studies. Embryogenic callus produces somatic embryos with globular structure while non embryogenic callus only show meristematic part without somatic embryos formation (Silveira *et al.* 2013). The absence of somatic embryo formation affected by lower cell differentiation related to protein degradation process and resulted in lower metabolic activity which inhibit somatic embryo development (Heringer *et al.* 2015). Somatic embryogenesis development required proper growth regulators to produce embryogenic callus. Plant growth regulators play a key role in both zygotic and somatic embryogenesis. Among all of them, auxin is the most effective for induction of somatic embryogenesis (Gulzar *et al*. 2020). Auxin reported to play important roles for callus induction, particularly 2, 4-D (Fig. 2) (Tahir *et al.* 2011). Addition of 2, 4-D was widely used for inducing rapid cell division and differentiation during callus induction in Gramminae family (Lee *et al.* 2012). High auxin concentration applied during induction stage; promote somatic cell to produce important substance for globular phase development in proliferation stage.

Previous studies stated that application of single 2, 4-D induced high performance of callus induction (Jahangir *et al.* 2010; Altaf *et al.* 2013). Induction media without 2, 4-D treatment mostly experienced slower callus growth even cell death (Alfian *et al.* 2019). Appearance of browning region supposed to be a limitation factor of lower callus

production. Phenolic compound from explant tissue inhibit nutrition absorption of explant, disturb enzyme activity and other metabolic process, and caused death of explants (Ahmad *et al.* 2013).

Application of plant growth regulator contributes to gene regulation during somatic embryogenesis initiation (Fig. 3). Genes involved during callus formation in sugarcane were SERK, BBM, and LEC. *Somatic Embryogenesis Receptor Kinase* (SERK) gene remarks embryogenic cell development and plays important role in early somatic embryogenesis pathway (Steiner *et al.* 2012). SERK gene was highly expressed in embryogenic callus of sugarcane. The expression patterns of SERK gene in sugarcane was similar to those described for the *Momordica charantia* (Talapatra *et al*. 2014). *Baby boom* (BBM) gene was responsible to promote the alteration of somatic cell. Over expression of BBM gene resulted in increasing SE development. Maulidiya *et al.* (2020) mentioned that BBM gene expressed at a high level in the globular stage and lower on the next proliferation stage. The expression of BBM gene was used as biomarker for SE initiation in *Theobroma cacao* (Florez *et al*. 2015). LEC also play important role in somatic embryo development and differentiation. Interestingly, our data revealed that WUS gene did not identified in embryogenic and non-embryogenic callus of sugarcane. It supported by that WUS gene regulation first localized not in callus tissue but in shoot meristem part of heart stage embryo. These finding justified the involvement of at least three genes during somatic embryogenesis induction in sugarcane. Expression of genes in embryogenic callus tended to support high production of somatic embryo through somatic embryogenesis pathway.

Six weeks callus induction considered as optimal age for transferring callus into proliferation media to produce somatic embryo. Proliferation is important stage for somatic embryogenesis development. Cell elongation occurs to develop somatic embryo during proliferation stage. This process involve role of 2, 4-D in lower concentration than induction to trigger continued cell division and produce somatic cell. Embryo somatic developments also require application of amino acid.

This research revealed the synergy of casein hydrolysate and proline combination for somatic embryo development. Both amino acids were very crucial for proliferation stage, particularly for increasing callus weight and number of coleoptile (Fig. 4). Amino acid such as casein hydrolysate and proline act as precursor for nucleic acids and other cellular process during somatic embryogenesis development. Casein hydrolysate applied as organic nitrogen sources for triggering callus growth and differentiation. Application of single casein hydrolysate could increase total callus weight per 100, even combination with proline showed higher total callus weight (Fig. 4A). Interestingly, presence of single casein hydrolysate only induced callus formation but did not promote callus differentiation until coleoptilar stage (Fig. 4B). This finding demonstrated that amino acid combination simultaneously supports somatic embryogenesis development. None coleoptile formed during late proliferation stage, expected as the result of failure callus growth due to the absence of L-Proline. L-proline promotes callus growth, enhance callus size and elongation (Kishor *et al.* 2015). However, higher than 500 mg L^{-1} proline concentration tended to decline total callus weight and number of coleoptile.

Development of somatic embryogenesis of sugarcane was recorded in this study. Best performance of somatic embryogenesis stages in each treatment during induction and proliferation (Fig. 5). Pro embryogenic mass (PEM) initiation occurred during 14 days after callus formation, followed by globular stage in next 14 weeks. Globular stage formed structure like embryo (embryoid) with two meristemoid regions (bipolar) (Pandey *et al.* 2018). Oliveira *et al*. (2017) claimed the existence of protodermal-dividing cell as competent region during morphogenetic process in somatic embryogenesis. This state contains high accumulated protein and starch contents for callus differentiation. It supported the finding of nodular structure after globular stage started which performed green spot, indicated meristematic tissue during scutellar stage. Green spot displayed faster in 7 days and appeared like heart structure. Cell elongation occurrence in late scutellar indicated shoot initiation and followed by coleoptile with bipolar structure after 26 days.

Coleoptile was the optimum stage used for embryo in synthetic seed encapsulation. Previous studies reported application of suitable coating material (*capsule*) such as sodium alginate and $CaCl_2$ required to produce and preserve synthetic seeds (Ali *et al.* 2012; Maqsood *et al.* 2012). The material of this capsule acts as an endosperm containing carbon sources, nutrients and growth regulators that affect the life of embryo, particulary on the viability of embryo in the seed.

Synthetic seeds germinate when the encapsulated embryos break the gel. This germination is influenced by the concentration of sodium alginate. The use of sodium alginate in different concentrations determines seed production and germination. Good synthetic seed characterized by compact, transparent, and firmly to wrap somatic embryo from mechanical damage (Gantait *et al.* 2015).

Seed germination of 3–4% sodium alginate proved to be better than 5% sodium alginate. Optimum concentration of sodium alginate promoted better shoot growth in regeneration stage. Concentration of 3 and 4% of sodium alginate together induced high number of shoot production. However, both concentrations resulted in different quantity and quality of plantlet. Plantlet in 3% of sodium alginate expected to provide optimum structure of capsule which is supported sugarcane embryo growth during germination stage. Higher concentration than 4% sodium alginate caused difficulty of the embryo to break the seed coat. It resulted in stunted growth, lower seed germination, and the seeds turned brown (browning) at regeneration stage.

Conclusion

Higher auxin concentration 4 mg L^{-1} was proper concentration to induce embryogenic callus production. Embryogenic and non-embryogenic callus formed during induction stage were regulated by somatic embryogenesis genes including SERK, BBM, and LEC. During proliferation stage, sugarcane callus required lower auxin concentration combined with proline and casein hydrolysate to increase cell growth and the number of coleoptile formed. Coleoptiles were selected as optimum stage for synthetic seeds embryo. Sugarcane coleoptiles were encapsulated in sodium alginate with 3% concentration for optimum embryo growth and development during germination stage.

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Author Contributions

PD, BS, and PO planned the experiments, AUKM and FNA interpreted the results, LIW and FNA made the write up and statistically analyzed the data and made illustrations.

Conflict of Interest

All authors declare no conflict of interest

Data Availability

Data presented in this study will be available on a fair request to the corresponding author

Ethics Approval

Not applicable in this paper

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