A SIMPLE PROTOCOL FOR SOMATIC EMBRYOGENESIS INDUCTION OF IN VITRO SUGARCANE (*Saccharum officinarum*. L) BY 2,4-D AND BAP

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ABSTRAK

Induksi tanaman tebu *in vitro* melalui teknik embriogenesis somatik sangat dipengaruhi dengan penambahan zat pengatur tumbuh. Tujuan penelitian ini adalah untuk menentukan formulasi medium yang tepat untuk induksi embriogenesis somatik tidak langsung pada dua jenis tebu potensial SUT *Event 02* dan PS 881. Penelitian terdiri dari tiga tahapan yakni induksi kalus, proliferasi kalus, dan regenerasi tunas. Bahan tanaman yang digunakan adalah pangkal batang planlet tebu SUT *Event* 02 dan PS 881 hasil regenerasi tunas sebelumnya berumur ± 1 bulan. Lima formulasi media yang berbeda digunakan pada tahap induksi kalus dan satu macam formulasi media pada tahap proliferasi kalus dan regenerasi tunas. Penelitian ini menggunakan Rancangan Acak lengkap (RAL) dengan lima macam konsentrasi media induksi yang berbeda. Hasil penelitian menunjukkan bahwa potensi tertinggi untuk regenerasi tebu *in vitro* SUT *Event 02* diperoleh dengan penambahan 2,4 - D 3 mgL^{-1} sedangkan potensi regenerasi tebu diperoleh dengan kombinasi 2,4-D 3 mgL^{-1} BAP + 1.5 mgL^{-1}pada varietas PS 881.

Kata kunci : embriogenesis somatik, kalus, 2,4-D, BAP

ABSTRACT

Induction of in vitro sugarcane through somatic embryogenesis technique influenced by addition of plant growth regulator. The objective of this research was to determine appropriate formulation medium for indirect somatic embryogenesis induction on two potential sugarcane SUT *Event 02* and PS 881. This research carried out in three steps, callus induction, callus proliferation, and shoot regeneration. Explants taken from basal of in vitro plantlet one month SUT *Even*t 02 and PS 881 resulted from shoot regeneration previously. Five different medium formulas applied for callus induction and one formula for proliferation and shoot regeneration. Research using completely randomized design (CRD) with five different formulation induction mediums. The result showed that concentrations of 2,4 - D with 3 mgL^{-1} provided high potential to regenerate in vitro sugarcane SUT *Event 02* while addition of a combination of 2,4-D 3 mgL^{-1} BAP + 1.5 mgL^{-1} provided high potential to regenerate PS 881 variety.

Key words: somatic embryogenesis, callus, 2, 4-D, BAP

INTRODUCTION

Somatic embryogenesis has great potential to be developed as excellent propagation technique in sugarcane (*Saccharum officinarum* L.*)*. Somatic embryogenesis is the formation process of embryo derived from the somatic cells or plant tissues grown at in vitro conditions and induced by externally plant growth regulators to support the growth of the explants into a new plant complete (Yuwono, 2008). Application of this technique helps to accelerate production a large number of ideal plant material for somatic seedling of sugarcane regeneration (Purnamaningsih, 2002) within short time.

Spesific stages of somatic embryogenesis development started from pro embryo mass formation followed by somatic embryo formation, maturation, and regeneration (Hussein *et al*, 2006; Purnamaningsih, 2002). The underlying principle of somatic embryo formation is totipotention phenomenon and has been reported extensively in some plant species of angiosperms and gymnosperm through plant tissue culture technique (Santos *et al.,* 2006) including in some sugarcane varieties (Raza *et al.,*2012*;* Malabadi *et al.*, 2011).

Formation of an embryo from a somatic cell can occur via two pathways, directly and indirectly. Direct embryogenesis (direct somatic embryogenesis) is formed of an epidermal cell explants without callus phase. While somatic embryogenesis indirectly (indirect somatic embryogenesis) is formed from the callus first, followed by embryo development of the section near the surface or in the callus. Direct somatic embryogenesis induction previously reported in rice using pro embryogenic cells from leaves, microspore, and protoplast without callus proliferation whereas indirect somatic embryogenesis were produced from friable embryogenic callus (Islam *et al.*, 2013).

Somatic embryo development is determined by a kinds of explant from part of plant. Plant regeneration using spindle leaf have been examined widely before in sugarcane (Mayang dkk., 2011; Anjum *et al.*, 2012) whereas Roy *et al.,* (2011) used immature leaf sheats of sugarcane as explant for callus production and somatic embryogenesis induction. Different studies have been repoted by Tiel *et al.,* (2006) and Mustafa and Khan (2012) using meristematic tissue from in vitro-grown plantlets from stem segments are taken at basal position up to the leaves for sugarcane regeneration. The highest regeneration efficiency was obtained from first cut mesristematic tissue from the basal stem in vitro planlets.

Regeneration and development of plant tissue of an explant is determined by exogenously applied growth regulators (Ali *et al.*, 2012). Application of auxin has important roles in cell growth, callus induction, root development, differentiation of vascular tissue, and cell diffusion. Previosly research has been reported by Naz *et al,* (2008) about application of combination between auxin and cytokinin in sugarcane induced the highest embryo somatic development. The type of synthetic auxin 2,4-D and cytokinin synthetic BAP applied to induce development of direct somatic embryo (Jahangir and Nasir, 2010). Application of single 2,4-D has high potential for callus initiation in sugarcane (Ali and Iqbal, 2010). Appropriate high concentration of 2,4-D in range 3-4 mgL^{-1} has been reported to obtain better respon in callus induction than low concentrattion of 2,4-D in range 1-1,5 mgL^{-1} (Ali *et al.*, 2010).

Application of plant growth regulator using BAP have been examined widely for regeneration of sugarcane (Sadat *et al.*, 2011; Ikram and Memon, 2012; Alcantara *et al*, 2014). Lower application of BAP induced good regeneration of sugarcane (Anjum *et al.*, 2012) whereas different concentration of BAP significantly affect to the presentase of callus regenration in sugarcane (Nawaz *et al.*, 2013). The objective of this research was to determine appropriate formulation medium for indirect somatic embryogenesis induction on two potential sugarcane SUT *Event 02* and PS 881.

MATERIALS AND METHODS

This research was conducted at the CDAST Laboratory, Division of Molecular Biology and Biotechnology, University of Jember from September 2014 - February 2015. This research used explants from basal segment of SUT Event 02 and PS 881 plantlets. Sugarcane in vitro plantlets one month old cut from 0.5 cm above the base stem. Basic media used for callus induction was Murashige and Skoog while the PGR used is 2,4 - Dicholorophenoxyacetic acid (2,4-D), Benzylaminopurine (BAP), casein hydrolyzate as an organic material , the amino acid proline , 3% sucrose , and phytagel 0.25 %. This research carried out in three steps, callus induction, callus proliferation, and shoot regeneration

Callus induction

Explant were cultured into sterile container contained medium supplemented by plant growth regulator treatments. Research using completely randomized design (CRD) factorial with five different formulation induction mediums, (1) MS (control); (2) 2,4-D 3 mgL^{-1}; (3) 2,4-D 4,5 mgL^{-1}; (4) 2,4-D 3 mgL^{-1} + BAP 1,5 mgL^{-1}; (5) 2,4-D 4,5 mgL^{-1} + BAP 1,5 mgL^{-1}. Each replication consists of three explants. The cultures placed in dark conditions to induce callus at a temperature of 23°C - 25°C for 5 weeks.

Data analysis performed using Analysis of Variance (ANOVA) for percentage of callus induction parameters. Highly significant parameters further tested using LSD. Data analyzed descriptively for callus development by macroscopic and microscopic observation using a stereo microscope.

Callus proliferation

Embriogenic callus from induction medium separated into smaller section and transferred to proliferation medium to induce development of somatic embryogenesis stages. The cultures placed in dark conditions to induce callus at a temperature of 23°C - 25°C for 4 weeks.

Regeneration of somatic embryo

Callus with well developed shoot system were tarnsferred to basic MS medium to promote development of whole part of plantlet. The cultures transferred in light conditions to induce plant development at a temperature of 23°C - 25°C .

RESULTS AND DISCUSSION

Days of callus initiation and callus percentage

The result of observation (Table 1) showed that explant swelling emerged at 2-4 days after planting and perfomed iniation callus sign at one week after planting. Rapid cells elongation during swelling process induced callus formation. Explants were grown in control media (MS) without the addition of PGR did not showed swelling respond within 30 days of incubation. Explants were grown in control media (MS) grew into shoots and roots until the end of observation at 30 days after planting. The growth indicated direct organogenesis without intervening callus phase. No number of callus were observed in explants and the value of data showed as not avalable (NA). Different respond showed in explants were grown in induction medium with addition of PGR whereas explants showed swelling respond.

Formation of callus were found different among the treatments. Development of explants by intervening callus phase were observed by recording days of callus initiation.

The result proved that addition of PGR affected to the days of callus initiation obviously. Basal explant of in vitro sugarcane mostly swelled particulary in the injured area (Figure 1). Explants on induction medium containing 2,4-D 3 mgL^{-1} in both SUT *Event 02* and PS 881 (A and C) showed more obvious alteration than explants on induction medium containing 2,4-D 4,5 mgL^{-1} (B and D).

The initial induction stage before the formation of callus, began with the swelling process in the basal of vitro explants precisely around the injured area. Basal section contain meristematic cell and produce the excellent response to callus (Mustafa and Khan, 2012). Swelling process in explants indicated rapid cell elogation (Gill *et al.*, 2004). PGR were added into medium become the major factor to coordinate cell division and explants morphogenesis (Figuroa *et al.*, 2006) and determine cell differentiation of plant tissue (Sukamadjaja and Mulyana, 2011).

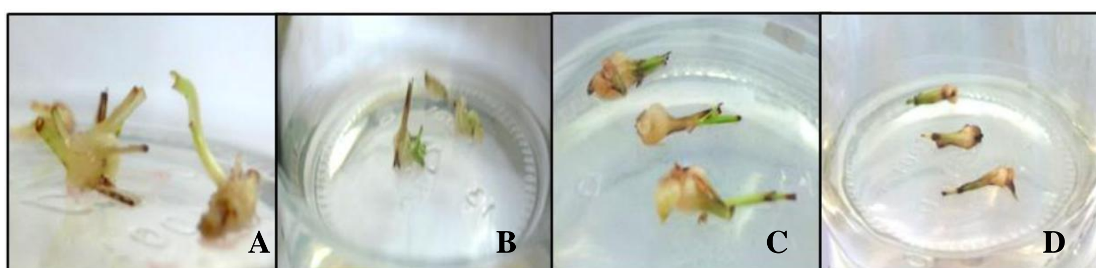

Figure 1. Basal explant of in vitro sugarcane at induction medium one week after planting. (A) Sugarcane SUT *Event* 02 at 2,4-D 3 mgL^{-1} (B) Sugarcane SUT *Event* 02 at 2,4-D 4,5 (C) Sugarcane PS 881 at 2,4-D 3 mgL^{-1} (D) Sugarcane PS 881 at 2,4-D 4,5 mgL^{-1}.

Based on the results in Table 1 showed that 2,4-D at 3 mgL^{-1} induced the fastest of callus initiation day than 2,4-D at 4,5 mgL^{-1} for both SUT *Event 02* and PS 881 although data showed not significantly different. Different results were obtained that addition of cytokinin BAP was not effective for callus initiation for both SUT *Event 02* and PS 881. Combination of BAP and 2,4-D on induction medium stimulated browing effect. Browning effect in the explants reduced cell division competence and inhibited callus formation of explants. Addition of 2,4-D and BAP into induction medium showed longer callus initiation day than single 2,4-D because of browning in the explants area.

Table 1. Days of callus initiation and callus percentage for two surgacane SUT *Event 02* and PS 881 under five different induction mediums.

Medium	Days of callus initiation (day)		Callus percentage (%)	
	SUT *Event 02*	PS 881	SUT *Event 02*	PS 881
A	NA	NA	0,71 a	0,71 a
B	9,30 a	7,00 a	10,02c	9,42 c
C	11,70 a	11,70 a	9,42 c	10,02 c
D	27,00 bc	27,00 bc	2,40 b	2,40 b
E	24,00 b	24,00 b	4,90 b	4,90 b

A= MS (kontrol), B= 2,4-D 3 mgL^{-1}, C= 2,4-D 4,5 mgL^{-1}, D=2,4-D 3 mgL^{-1} + BAP 1,5 mgL^{-1}, E= 2,4-D 4,5 mgL^{-1} + BAP 1,5 mgL^{-1}.
NA = Not available. Different letter are statically different significant (P<0,05%).

Best response for percentage of callus were obtained in 2,4-D treatment at both SUT *Event 02* and PS 881. Callus induction at 2,4-D 3 mgL^{-1} and 4,5 mgL^{-1} level showed higher percentage than combination of 2,4-D and BAP. Addition of PGR at 2,4-D 3 mgL^{-1}

and 4,5 mgL^{-1} level were optimum for callus indution of sugarcane SUT *Event 02* and PS 881. In contrast with previous research stated that combination of auxin and cytokinin most effective for somatic embryo induction (Jahangir and Nasir, 2010), in this research showed combination formula were not effective for callus initiation whereas callus initiation is the main process to promote somatic embryo production. Callus from the end of induction stage, were subcultured into proliferation medium particulary from B, C, and E mediums at both SUT *Event 02* and PS 881. Explants from A medium were not subcultured into proliferation medium because explants showed direct organogenesis response whereas explants from D medium could not be subcultured because callus not developed properly.

Callus morphology

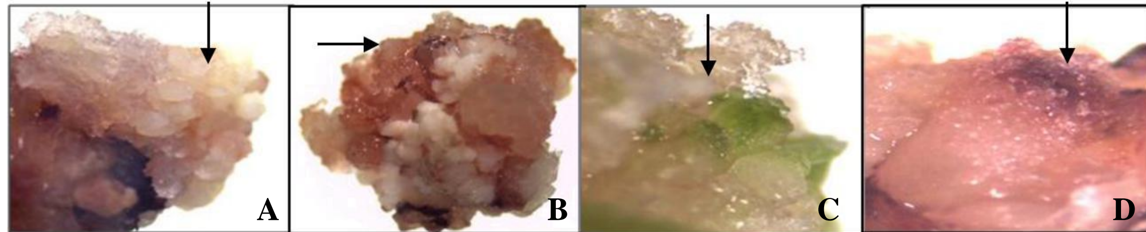

Figure 2. Callus morphology in sugarcane (A) Embryogenic callus of SUT *Even*t 02 (B) Non embryogenic callus of SUT *Even*t 02 (C) Embryogenic callus of PS 881 (D) Non embryogenic callus of PS 881.

Based on the results of microscopic observation different characteristics of callus were observed between SUT *Event 02* and PS 881 (Figure 2). Types of callus were identified as embryogenic and non embryogenic. The characteristic of embryogenic callus were friable, glossy, round and richly cytoplasmic cells (Figure 2A and 2C) whereas non embryogenic callus were creamy-yellow in colour, compact, watery, and slighty browned (Figure 2B and 2C). Embryogenic callus in PS 881 showed glossy callus and emerged green spot where the presence of green spots generally will grow into leaves and stems, while the characteristics of the callus non embryogenic has the same features as callus contained in sugarcane SUT *Event 02.* Non embryogenic callus were separated with embryogenic section, then were subcultured to optimize embryogenic cells development. Competent embryogenic callus with furthermore will develop into a unit that resembles an embryo (embryoid) which has two candidates meristem (bipolar) which will then be passed through the stage of maturation and germination after sub cultured on proliferation medium . The observation of callus at the end of the induction phase showed that callus cultured in B, C, and E medium for sugarcane SUT *Event 02* exceeded for the next proliferation stage.

Callus morphology obtained at induction stage, was observed particulary about embryogenic and non embryogenis characteristic microscopicly using stereo microscope. Embriogenic callus is a type of callus which can produce somatic embryo after addition of treatment in culture medium (Hussein *et al*, 2006). Development of embryogenic and non-embryogenic callus related with internal factor and differentiation of each plant tissue (Figuroa *et al*. 2006).

Callus morphology in proliferation stage

The results of the proliferation stage after two weeks incubation showed of presence of somatic embryogenesis stages from both sugarcane SUT *Event 02* and PS 881.

The morphology of embryogenic stages were found up to 4 weeks using a stereo microscope. Somatic embryogenis stages in both SUT *Event 02* dan PS 881 include globular, scutellar, coleoptilar dan cotyledon and observed on the embryogenis callus surface. These stages occured in the short time and their ability decrease as the long duration of incubation medium (Roy *et al*., 2011).

Embryogenic cells from callus induction furthermore stimulated for developing somatic embryos. Embryogenic callus were sub cultured into proliferation medium containing 2,4-D 1,5 mgL^{-1} + prolin 560 mgL^{-1}+ casein hidrolisat 300 mgL^{-1}. Proliferation medium containing lower concentration of 2,4-D as reported by Jimènez, (2001) and Figuroa *et al*., (2006) to optimize development of somatic embryogenesis stage. Prolin in proliferation medium were added to increase somatic embryogenesis in sugarcane (Gill *et al*., 2006; Roy *et al.,* 2011).

Callus morphology observation focused on observing embryogenic callus characteristic. Embryogenic callus will develope into somatic embriogenesis stages. Development of somatic embryos were produced from embriogenic callus in proliferation medium. Early stages of embryo formation began with the formation of pro embryo mass (PEM) or pro globular with glossy and friable structure. In this research, pro embryo mass (PEM) of SUT *Event 02* and PS 881 will further develop into nodular embryogenic callus-shaped, glossy, crumbs, dry, and transparent. Incubation stages in proliferation medium triggered pro- embryonic structures in embryogenic callus and then developed into somatic embryos through the stages - such as the formation stages of zygotic embryos.

Globular stage emerged after the cell division which is characterized by nodular structure and has suspensor. Nodular callus will further develop into terminal bud (terminal leaf node) in the form scutelum characterized by scutelar node. Scutelum consist of cells rich in cytoplasm with irregular shape.The development of scutelum into coleoptile marked by the increasing layers of cells to form coleoptile (Alcantara *et al.,* 2014). Coleoptile development of callus can be used as an encapsulation material for the production of synthetic seeds of sugar cane. Coleoptile stage has the potential to serve as an encapsulation material for synthetic seed due to be meristematic. Meristematic nature has the potential to support the development of plant tissue to be complete plants. Development stage of the cotyledon is the result of coleoptile development stage in the late stages of maturation where coleoptile seemed directed its development into a complete structure with prospective shoots and roots (bipolar) (Purnamaningsih, 2002).

In this study, callus from B and C induction medium for SUT *Event 02* allowed to transfer into regeneration medium while callus from E medium did not show good potential to regenerate. Unlike PS 881, the result showed callus from B and C induction medium did not develop into phases - phases of somatic embryogenesis and regeneration ability less than the maximum. Callus from D and E medium for PS 881 showed good response for shoot development and had potential to transfer into regeneration medium.

In this research, sugarcane SUT *Event 02* and PS 881 had different specific characteristics. Each clumps of callus on the proliferation medium had different response - depending on the growth of shoots either from sugarcane SUT *Event 02* or PS 881. Candidates shoots that grow from callus then transferred to regeneration media containing only MS (Murashige and Skoog) without the addition of growth regulator substances. Potential regeneration of callus is highly dependent specific genotypes along with a combination of concentration and composition in regeneration medium (Behera and Sahoo 2009).

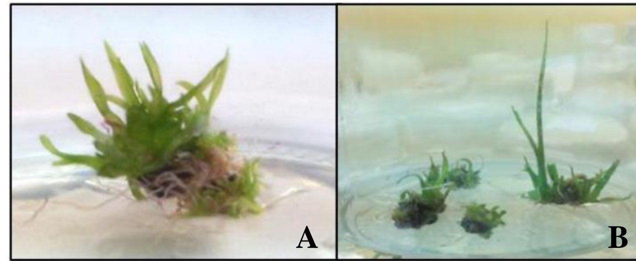

Figure 3. Regeneration of somatic embryo from SUT Event 02 and PS 881 (A) SUT *Even*t 02 (B) PS 881.

The present study produced plants regenerated through two different pathways that direct somatic organogenesis and indirect somatic embryogenesis. Based on the observations of regeneration phase, showed that callus induction medium derived from B medium (2,4-D 3 mgL^{-1}) for SUT *Event 02* showed the best regeneration potential than other induction media origin (Figure 3A). Callus induction from media origin C (2,4 - D 4.5 mgL^{-1}) can qualify up to the regeneration stage and have the potential to develop into embryogenic callus regeneration but lower than the original callus induction medium B while the origin callus induction media D and E do not qualify until the regeneration step. Different results was obtained from PS 881 where callus from induction medium D and E did not showed good result in induction and proliferation medium but showed best results for shoot regeneration (Figure 3B). Day of shoots formation from coleoptile stage already indicated that the meristematic explants properties germinated in optimum condition. Good plant growth response in the regeneration medium was characterized by the ability of plants to produce new shoots that indicate the plantlets can grow well .

CONCLUSION

The result showed that the best response of indirect somatic embryogenesis on SUT *Event 02* and PS 881 was medium containing 3 mgL^{-1} of 2,4-D. Concentrations of 2,4 - D with 3 mgL^{-1} provided high potential to regenerate in vitro sugarcane SUT *Event 02* while addition of a combination of 2,4-D 3 mgL^{-1} BAP + 1.5 mgL^{-1} provided high potential to regenerate PS 881 variety.

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