INDUKSI KALUS DAN DAYA REGENERASI *IN VITRO* BERBAGAI UMUR KALUS DAN KULTIVAR TEBU THAILAND (*Saccharum officinarum* L.)

CALLUS INDUCTION AND PLANT REGENERATION ABILITY OF VARIOUS CALLUS AGES OF CERTAIN THAI SUGARCANE CULTIVARS

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FAKULTAS PERTANIAN
UNIVERSITAS JEMBER
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SKRIPSI

Diajukan guna melengkapi tugas akhir dan memenuhi salah satu syarat
untuk menyelesaikan Program Studi Agroteknologi (S1)
dan mencapai gelar Sarjana Pertanian

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2015

PERSEMBAHAN

Dengan memanjatkan puji syukur kehadirat Allah Subhanahu wa ta'ala, skripsi ini saya persembahkan untuk :

1. Ayahanda Gatot Subagio dan Ibunda Sudi Rahayu tercinta, kuhaturkan terima kasih atas segala pengorbanan, kasih sayang, dan doa yang selalu dipanjatkan,
2. Adik-adikku tersayang dan keluarga besar yang selalu memberi semangat dan doa,
3. Semua guru dan dosen sejak taman kanak-kanak hingga perguruan tinggi,
4. Almamater Fakultas Pertanian Universitas Jember.

MOTTO

"Dan di bumi ini terdapat bagian-bagian yang berdampingan, kebun-kebun anggur, tanaman-tanaman dan pohon kurma yang bercabang dan yang tidak bercabang, disirami dengan air yang sama. Kami melebihkan sebahagian tanam-tanaman itu diatas sebahagian yang lain tentang rasanya. Sesungguhnya pada yang demikian itu terdapat tanda-tanda (kebesaran Allah) bagi kaum yang berfikir"

(terjemahan Surat *Ar-Ra'd* (13) ayat 4)[*)]

"*Sesungguhnya Allah tidak akan mengubah nasib suatu kaum, kecuali kaum itu sendiri yang mengubah apa-apa yang ada dalam diri mereka*"

(terjemahan Surat *Ar-Ra'd* (13) ayat 11)[*)]

[*)]Departemen Agama Repuplik Indonesia. 1998. *Al Qur'an dan Terjemahannya*. Semarang: PT. Kumudasmoro Grafindo.

SURAT PERNYATAAN

Saya yang bertanda tangan dibawah ini :

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menyatakan dengan sesungguhnya bahwa karya tulis ilmiah yang berjudul : **"Induksi Kalus dan Daya Regenerasi *In Vitro* Berbagai Umur Kalus dan Kultivar Tebu Thailand (*Saccharum officinarum* L.)"** adalah benar hasil karya sendiri, kecuali disebutkan sumbernya dan belum pernah diajukan pada institusi manapun, serta bukan karya jiplakkan. Saya bertanggung jawab atas keabsahan dan kebenaran isinya sesuai dengan sikap ilmiah yang harus dijunjung tinggi.

Demikian surat pernyataan ini saya buat dengan sebenarnya, tanpa adanya tekanan dan paksaan dari pihak manapun serta bersedia mendapat sanksi akademik jika ternyata di kemudian hari pernyataan ini tidak benar.

Jember, 12 Mei 2015

Yang menyatakan,

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CALLUS INDUCTION AND PLANT REGENERATION ABILITY OF VARIOUS CALLUS AGES OF CERTAIN THAI SUGARCANE CULTIVARS

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SPECIAL PROBLEM
BACHELOR OF SCIENCE
DEPARTMENT OF HORTICULTURE

CALLUS INDUCTION AND PLANT REGENERATION ABILITY OF VARIOUS CALLUS AGES OF CERTAIN THAI SUGARCANE CULTIVARS

By

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RINGKASAN

Induksi Kalus dan Daya Regenerasi *In Vitro* Berbagai Umur Kalus dan Kultivar Tebu Thailand (*Saccharum officinarum* L.); Rahmat Budiarto; 111510501106; Program Studi Agroteknologi; Fakultas Pertanian; Universitas Jember.

Sebagian besar (70%) gula yang digunakan secara global diproduksi dari usaha tani tebu. Peningkatan kuantitas dan kualitas produksi gula tebu dapat dilakukan dengan kultur jaringan. Kultur jaringan adalah teknik mengkulturkan sel dan jaringan terpisah dari tanaman inang, ditumbuhkan dalam tabung dengan media bernutrisi dan mikroklimat terkondisikan. Keberhasilan kultur jaringan ditentukan oleh daya regenerasi kalus. Daya regenerasi kalus pada kultivar dan umur yang berbeda juga tidak akan seragam. Sebagian besar peneliti mengkultur kalus tidak lebih dari dua bulan, karena semakin tua kalus, semakin banyak sel penyusun kalus yang telah kehilangan daya regenerasi. Tujuan dari penelitian ini adalah (i) untuk mengetahui karakteristik kalus pada berbagai umur dan kultivar tebu, (ii) untuk mengetahui interaksi umur kalus dan kultivar terhadap daya regenerasi *in vitro*, (iii) untuk mengetahui kultivar tebu regeneratif *in vitro* jangka panjang

Penelitian ini dilaksanakan di Center of Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen campus, Thailand selama enam bulan, mulai bulan November 2013 – Mei 2014. Penelitian ini terdiri dari dua tahap yaitu induksi kalus dan regenerasi tunas. Penelitian ini menggunakan Rancangan Acak Lengkap dengan dua faktor dan sepuluh ulangan. Faktor pertama adalah kultivar tebu dengan 4 taraf yaitu LK 95-127, LK 92-17, K 93-219, dan K 93-347. Faktor kedua adalah umur kalus dengan tiga taraf yaitu dua, tiga dan empat bulan. Induksi kalus dilaksanakan dalam ruang gelap bersuhu 27^0C selama empat bulan dengan subkultur setiap bulan. Kultur regenerasi tunas berlangsung selama dua bulan dalam ruang kultur terang bersuhu 27 ± 2^0C. Variabel pengamatan meliputi persentase kalus embriogenik, diameter kalus, warna kalus, struktur kalus, persentase regenerasi tunas, jumlah tunas, dan tinggi tunas. Warna dan struktur

kalus diamati secara visual dan disajikan dalam bentuk gambar. Variabel pengamatan lainnya dianalisis dengan sidik ragam dan apabila terdapat beda nyata dilanjutkan dengan uji Duncan pada taraf kepercayaan 95%.

Sebagian besar kultivar mampu menghasilkan 100% kalus embriogenik terkecuali LK 92-17. Karakteristik kalus tebu embriogenik adalah berwarna kuning keputihan, tidak basah, struktur kompak dengan tonjolan nodular. Kalus embriogenik lebih mudah diregenerasikan dari pada kalus non embriogenik yang dicirikan dengan warna abu-abu, basah, dan tidak menampakan nodular yang jelas. Karakteristik tambahan untuk kultivar K 93-347 adalah adanya sekresi senyawa fenol yang berlebih sehingga menyebabkan *browning*. Pada variabel pengamatan diameter kalus, kultivar K 93-347 menghasilkan kalus dengan rerata diameter terbesar sedangkan LK 92-17 menghasilkan kalus dengan rerata diameter terkecil. Hasil penelitian pada variabel pengamatan persentase regenerasi tunas menunjukan bahwa setiap perlakuan dapat diregenerasikan secara menyeluruh (100%) dalam kurun waktu dua bulan. Tunas terpanjang dihasilkan oleh kombinasi perlakuan umur kalus dua bulan pada kultivar K 93-219 dan K 93-347. Jumlah tunas terbanyak dihasilkan oleh kombinasi perlakuan umur kalus dua bulan pada kultivar LK 95-127. Kultivar yang direkomendasikan sebagai kultivar regeneratif *in vitro* jangka panjang adalah K 93-347 karena daya regenerasinya dapat bertahan baik dengan indikator persentase regenerasi tunas mencapai 100% dan jumlah tunas yang dihasilkan oleh kalus tertua (empat bulan) berbeda tidak nyata dengan kalus yang muda (dua dan tiga bulan).

SUMMARY

Callus Induction and Plant Regeneration Ability of Various Callus Ages of Certain Thai Sugarcane Cultivars; Rahmat Budiarto; 111510501106; Study Program of Agrotechnology; Faculty of Agriculture, Jember University

Most of global sugar production (70%) derived from sugarcane plantation. Sugarcane, as an agricultural comodity with global importance, stymulate many research related to quantity and quality improvement through tissue culture. Tissue culture is the cultivation of cells and tissues separate from the organism and place on formulated nutrient medium under human controlled on the right conditions. The success of tissue culture is determined by regeneration ability of callus. Regeneration is varies on various callus ages and cultivars. Most of researchers culture callus less than two month, since the older callus, the more cells lost their regenerable properties. This research aimed (i) to determine callus characteristics at different ages and cultivars of sugarcane, (ii) to determine the interaction between callus age and cultivar on regeneration ability, and (iii) to determine the long term in vitro regenerable cultivar.

The research was conducted from November, 2013 to May, 2014 at Center of Agricultural Biotechnology (CAB), Kasetsart University, Kamphaeng Saen campus, Thailand. The experiment composed of two stages, callus induction and shoot regeneration. Experimental design used was completely randomized design (CRD) with two factors. The first factor was sugarcane cultivars (v) comprised of four levels, namely: LK 95-127, LK 92-17, K 93-219, and K 93-347. The second factor was callus ages (u) which consisted of three levels, namely two months, three months, and four months. All treatments replicated ten times. Callus cultures were incubated under dark condition at regime 27^0C and run up to four months with monthly subcultured onto fresh CIM. Regeneration cultures were incubated under light condition at regime 27 ± 2^0C within two months with monthly subcultured. Several variable observed were percentage of embryogenic callus, colour, structure, and diameter of callus, percentage of shoot regeneration, the number of and lenght of shoot. Physical appearance, such colour and structure,

was observed and depicted in form of tables and photos. Other variables were analyzed using analysis of variance (ANOVA). The significant differences between treatments was analyzed by Duncan's Multiple Range Test at 5 % significant level.

Most of treated cultivars produced 100% of embryogenic calli, except LK 92-17. Embryogenic callus characteristics were whitish yellow, dry and compact with nodular structure. Embryogenic callus were easier to regenerate than non embryogenic callus. Additional characteristics only for K 93-347 was excessive production of phenolic compound that indicated by brown colour in growing media. K 93-347 produced the widest diameter calli than others. All treated calli could be regenerated into shoot within two months. The longest shoot was produced by combination of two months callus ages with K 93-219 and K 93-347. The highest number of shoot was produced by combination of two months callus ages with LK 95-127. The K 93-347 was determined as long term in vitro regenerable cultivar, since it had good and stable regeneration performances indicated by 100% of shoot regeneration and the number of shoot produced by oldest calli (four months) was not significantly different with young calli (two and three months).

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I. INTRODUCTION

1.1 Background

Sugar is the most popular sweetener in the term of global consumption and trade. About 82 % of global sweetener derived from sugar (Taylor, 2013). Khan and Katri (2006) reported that 70% of global sugar production derived from sugarcane plantation. Sugarcane is cultivated in over 120 countries of the tropics and subtropics (Gallo-Magher *et al.*, 2000). Thailand is the highest rank of sugarcane production around Southest Asia and become the 2nd sugarcane exporter around the world after Brazil. High sugarcane demand around Asia, particularly from Indonesia and Cambodia, stymulated Thailand to exports around 8.7 million tons (November 2013/14), estimated by USDA (2013). The consumption pattern of sugarcane increased because of improving number of household and industry. USDA (2013) recorded that Thailand sugarcane production was estimated up 900,000 tons, to a record 10.9 million tons based on a rise in area and yield.

Rapid production of sugarcane should be supported by high quality and quantity of plant material. Most of sugarcane is clonally propagated from which multiple annual cuttings of stalks are typically obtained from each planting (Ather *et al.*, 2009). The drawbacks of plant material derived from cutting are less performances of root and highly depend on the growing season. The conventional propagation methods is also high susceptibility to plant disease and virus contamination. High quality of sugarcane can be achieved through conventional breeding and also genetic tranformation. Conventional breeding takes long time and has some limitation of certain traits. Genetic transformation is the insertion of foreign and new genetic material (interest gene) into plant cells in order to improve the performance and its productivity. Genetic transformation and plant propagation in sugarcane highly rely on tissue culture.

Tissue culture is the cultivation of cells and tissues separate from the organism and place on formulated nutrient medium under human controlled on the right conditions. . Goerge *et al.* (2008) defined plant tissue culture as the

science of growing plant cells, tissues or organs isolated from the mother plant, on artificial media. Tissue culture is important technology for production of disease-free planlets, high quality planting material and the rapid production of many uniform plant (mass-micropropagation). The advantages of tissue culture are no interfering large portion of genome, true-to-type plants, high number of planlets production during short time, more effiency in term of labor, money, and place.

Some stages during tissue culture is usefull for genetic transformation and disease free planlets production. Since, many researchers assemble genetic transformation in callus stage, for example applying particle bombardment on embryogenic callus in order to get sugarcane resistant to *Sugarcane Mosaic Virus* (Ingelbrecht *et al.*, 1999) and resistant to herbicides (Gallo-Meagher *et al.*, 2000). *Sugarcane Yellow Leaf Virus* (SCYLV)-free infected planlets had obtained from tissue culture by Comstock and Miller (2004). High frequency of regeneration system is very important for production of transgenic plant (Raja *et al.*, 2009).

Mostly researchers who used in vitro selection rather than field selection should capable to maintain their callus in regenerable state at least equal to the selection period or even longer. The advantages of using in vitro selection are environmental factors under human control and allows the selection at the cellular level. Selection of interest gene and also verification of virus-free material in callus stage takes time depend on the method. Vyver *et al.*, (2013) takes 15 weeks for selection of sugarcane to herbicides. Purnamaningsih *et al.*, (2013) takes three months that two months for pre-selection after mutation planted on the same induction medium and one months later for selection.

Regeneration ability is depend on callus ages and genotype. Callus age is a time span ranging from callus was formed until decline or death. Normally, researchers cultured callus in growing medium with high auxin content not more than two months since the decline of regeneration ability showed after long term cultures. Anjum *et al.*, (2012) used only 21, 28 and 35 days-old sugarcane calli for in vitro regeneration. The research conducted by Tiwari used two cultivar namely Cos 8820 and Cos 767 reported that 60% of 15 weeks calli death and cannot regenerate. Fiah *et al.*, (2014) reported that 12 weeks culture in high auxin

growing medium and three times subcultured caused the death of callus tissues indicate by black tissue colour and lost of regeneration ability. Anjum *et al.* (2012) reported that sugarcane genotype S-2003-us-127 showed the best regeneration by using 28 days old calli, whereas S-2003-us-371 showed the best performances if only used 35 days old calli. It is strong evidence that genotype also play great role to determine regeneration ability. There is rarely to find any research that studied regeneration ability of callus over two months on certain high yielding cultivars of Thai sugarcane.

1.2 Problem Identification

High yielding sugarcane cultivars potential to propagate rapidly through tissue culture. Callus is raw material for regeneration planlets. Callus culture require high concentration of 2,4 D as synthetic auxin. Most researchers cultured callus less than two months to avoid the lost of regeneration ability. Several circumstances required callus that kept regeneration ability longer such three and four months. The study of regeneration ability on various callus ages derived from certain high yielding sugarcane cultivars is needed.

1.3 Objectives

a. To determine callus characteristics at different ages and cultivars of sugarcane.
b. To detemine the interaction between callus ages and sugarcane cultivars on regeneration ability.
c. To determine the long term in vitro regenerable cultivar.

1.4 Benefit

a. To provide information for sugarcane breeder deals with callus age and cultivar that well-suited for genetic transformation and disease-free planlets production related with their regeneration ability.
b. To provide the information needed to plan all year round availibility of regenerable callus for production of planlets.

II. LITERATURE REVIEW

2.1 The Importance of Sugarcane

World sugar production is less than with the amount of consumption. According to Illovo (2012), global sugar production in 2011/12 was forecasted at 179 million tons, representing 7% increase compared to that year of previous year. In other side, global sugar consumption growth during 2011/12 is increased by 1.5% due to greater sugar availability and lower prices than that of the previous year.

Sugar production mostly rely on sugarcane farming sector. Sugarcane is cultivated around the world, both subtropics and tropics. Southest Asia and Southern America is the best region in the term of sugarcane production. The most highest rank of sugarcane production around Southest Asia and the second sugarcane exporter around the world after Brazil is Thailand. High sugarcane demand around Asia, particularly from Indonesia and Cambodia, stymulate Thailand to exports around 8.7 million tons (November 2013/14), estimated by USDA (2013). The pattern of Thailand's sugarcane exports during the last five years shows the improvement. USDA (2013) recorded that Thailand sugarcane production is estimated up 900,000 tons, to a record 10.9 million tons based on a rise in area and yield. The consumption pattern is also going up, because of improving number of household and industry.

Indonesian sugar production is not comparable with the amount of consumption. Based on data from the Association of Indonesian Sugar (IKAGI), consumption of sugar production in January - October 2011 amounted around 2.11 million tonnes. In other hand, domestic consumption of sugar around 2,7 - 2,8 million tons/year (Julian, 2011). The less sugar production caused by the decreasing of sugarcane planting area. According to the Indonesian Sugar Council (DGI) since the 2008-2010 decline in sugar cane planting area of 436 504 ha (2008) to 422 935 ha (2009), and in 2010 to 418 259 ha (Julian, 2011).

2.2 Sugarcane Cultivar

Sugarcane (*Saccharum officinarum* L.) is a perennial grasses in kingdom *Plantae*, family *Poaceae* and genus *Saccharum* originally from warm temperate to tropical regions. It posses unique characteristic such as tall, thick, solid, juicy and high concentrations of sucrose in stems. That is why it mostly used for basis of sugar production. Sugarcane properties such as an efficient photosynthesis and efficient biomass production make this an excellent target for industrial processing, a valuable alternative for animal feeding, the production of by-products, and a prime candidate as a fuel crop for production of ethanol (Ather *et al*.,2009).

Generally morphology of sugarcane is not much different from plants in family *Poaceae*. The root system is fibrous and composed of adventitious and permanent root. Advetitious root comes first from root primordia in nodes of cane setts and serves as water absorbtion unit during sprouting and waiting for permanents root formed. Permanent roots, also know as true roots, emerge from root primordia in bottom part of shoot itself and the function is nearly the same as usual root such as keep the cane upright on soil, attacking and supply of water and nutrients from the soil.

The cane is tall, thick, round, solid, juicy and contains high concentrations of sucrose. The colour of cane is varies form green upto red purple. As nature of grass family, the cane composed of alternating nodes and internodes. Internodes is a part of stem elongation between two nodes and are usually found much accumulation of sucrose. Nodes are points where the leaves attach and normally each node posses a leaf scar, axillary bud and axillary root primordia. In addition, nodes is equipped by intercallary meristem as a growth ring (Australian Goverment, 2008) used to increase of internodes length.

The leaves are ligulate like a long ribbon. They are arranged alternately along the stem. One nodes composed of one leaf which divided into blade and sheath. Leaf blade alike a long ribbon stretched with a pointed end, fluffy, green, and parallel veins.

The inflorescence type of sugarcane is ramified. The last stalk internodes growth continue to be conoidal panicle with a main axis called the rachis. Rachis holds secondary branches. Secondary branches holds tertiary branches. At the base of the tertiary branches, it is a location of the spikelets. The spikelets also located on the top of the secondary branches. The spikelet is the basic unit of the grass inflorescence. Each spikelet has one flower, which is sit in rows along the inflorescence secondary and tertiary branches. Sugarcane flower isnot belong to complete flower because of donot have callyx, only have crown, stamen and pistil. Sugarcane flower has two crowns, the bottom called lemma and the other being called palea and also has two lodicula located on the base of the flower, which is actually two petals in a changed form. Sugarcane flowers is a perfect flower (hermaprodites), which are have both male flower (stamen) and female flower (pistil) in one flower (it also called monoecious). Sugarcane flowers consist of three stamens and one pistil.

Sugarcane is a clonally propagated crop from which multiple annual cuttings of stalks are typically obtained from each planting (Ather *et al.*, 2009). Propagation of sugarcane is generally done vegetatively through cuttings. But mostly people find many disadvantages of sugarcane cuttings using planted material. The drawbacks such as the longer time used, requiring a lot of parent plant and energy, difficult to avoid pathogen contamination, and highly depend on the growing season (Sukmadjaja and Mulyana, 2011).

Cultivar is stands for cultivatated variety, defined as a group of plants which are distinct from the others, but uniform within its group and stable in expresion of economic characters. Cultivars were divided into 4 types, namely open pollinated varieties, pure line or inbred line varieties, hybrid varieties and clones. Open pollinated varieties is one in which pollination is carried out by wind, insects, or other naturally occurring agents and indicated by genotypic frequency p2AA, 2pqAa, q2aa. This variety donot undergone selection, mutation, migration random drift within population. It is very constrast with controlled pollination whereby breeder try to ensure that all sedds they obtained is come from known parent and rationale to obtain the desired traits from its parent. Pure

line is the varieties derived from long selfing in self-polliated crops, otherwise inbred line is the varieties obtained from long selfing in cross-pollinated crops. Hybrid varieties is the varieties with superior agronomic traits due to hybrid vigor, derived from single cross, three-ways cross or either double cross hybrid. Clones is the line which derived from vegetative propagation. Those basic classification is ease the researchers to determine and collect sugarcane plant material in their research.

Modern sugarcane cultivars are highly polyploid and aneuploid (2n = 100–130) derived from inter-specific hybridization between ancestral polyploid species, namely *Saccharum officinarum* L. and *Saccharum spontaneum* L. (Andru *et al.*, 2011 ; Raboin *et al.*, 2006). This genetic structure inhibit researchers to fully map the whole genome of sugarcane. Its size and complexity represent a major challenge for the isolation of agronomically important genes (Cunff *et al.*, 2008). The genome of sugarcane may be the same among the various cultivar. But the genetic make-up will be slightly different.

There are several consideration used to determine cultivars for present research such as yield, comercial cane sugar, harvesting age, ratooning, tillering, and flowering. Yield is the most economically determination point of sugarcane plantation. Yield fully measured by weighing the wet cane directly harvested from the sugar cane fields. Harvesting age is related to the readiness of cane to be harvested according to requirements of factories. *Commercial cane sugar* (*CCS*) is calculated from Brix, Pol and fiber content of sugarcane and estimates the level of extractable sucrose minus a negative weighting based on the level of impurities at harvest (Albertson and Grof, 2004). CCS become the determinant factor deals with the payment of cane yield to farmers. Flowering is undesirable traits in case of sugarcane for farmers in stead of plant breeders. In case of plantation, sugarcane should be spared from flowering due to the decline of CCS on final yield when flowering comes. The best cultivars requires no flowering.

Ratooning is a method of harvesting sugarcane by leaving the lower parts and roots of this plant in order to have the stubble crop as the plant material for the next season. The benefit of ratooning is early matures and save the cost of land

preparation and planting. The disadvantage is thinner canes than before, with low sugar content. As consequently, total yield will decreases after each cycle. Not of all sugarcane cultivars have good ratooning. Good ratooning means the ability to produce a steady yield for 5-6 season. Longer ratooning also increase the proportion of land in effective use in farm, which results in a large economic gain and increased competivity, mainly in areas of strong competition for land with other crops or land diversion for other more value-added purpose (Matsuoka and Stolf, 2012).

Tillering refers to the ability to produce of side shoots and related to vegetative propagation. Tillering is natural property of *Poaceae*, include sugarcane. Sugarcane should have optimum tillers in order to improve yields. Tillering is a primordial characteristic of sugarcane because the main sink of the product of photosynthesis are the stalks formed from growth of the tillers and thus, the profitability of the crop depends primarily on the tillers produced that will dictate the final numbers of harvestable stalk (Matsuoka and Stolf, 2012). The best cultivars in the case of tillering is cultivars with up to 4 tillers.

Table 2.1 Selected sugarcane cultivars from Sugarcane Research Center in Kasetsart University at Kamphaeng saen campus

Agronomy Traits	Cultivars			
	LK 92-17	LK 95-127	K 93-219	K 93-347
Yields (ton/rai)	15-18	18	16-21	16-20
CCS	11-13	13	12-14	11-13
Harvesting age (months)	10-12	12	12	12
Ratooning	Good	Good	Good	Good
Tillering	Moderate (4-5 stalks/plant)	Moderate (4-5 stalks/plant)	Moderate (4-5 stalks/plant)	Moderate (4-5 stalks/plant)
Flowering	Few	No	No	No

Source : Chanprame (2014).

Sugarcane cultivars used to date comes from Sugarcane Research Center in Kasetsart University at Kamphaeng saen campus. These research center integrated with sugarcane germplasm that composed of almost all varieties grown

in Thailand. Each cultivar is cultivated parallel and constitute 5 line rows. The database of names and agronomic characters of each cultivars are saved and provided for research interest. Basicly, we should choose any cultivar with good agronomic traits. Based on these consideration, there are 4 cultivars namely LK 92-17, LK 95-127, K 93-219 and K 93-347 with desirable traits as depicted in Table 2.1.

2.3 Tissue Culture of Sugarcane

Tissue culture is the cultivation of cells and tissues separate from the organism and place on formulated nutrient medium under human controlled on the right conditions in order to obtain planlets. Goerge *et al.* (2008) defined plant tissue culture as the science of growing plant cells, tissues or organs isolated from the mother plant, on artificial media. There are so many advantages of tissue culture, such as no interfering large portion of genome, true-to-type plants, high number of planlets production during short time, more effiency in the term of labor, money, and place.

The basic concept of tissue culture is totipotency as Motte *et al.*, (2014) asserts that the remarkable ability of plant cells to regenerate can be traced back to the totipotency theory first hypothesized by Haberlandt at 1902 : " Theoretically all plant cells are able to give rise to a complete plant". Totipotency related to ability of cells to undergone differentiation. Totipotency of cell can stymulate forming of the entire planlets through regeneration. The younger tissue used for explants, the more efficient callus induction since its high potentiality to undergone differentiation.

The conventional methods sugarcane propagation that used cutting as planted material is high susceptibility to virus. The most treatment used in conventional methods is Moist Hot Air Treatment (MHAT) which is not effective to against mosaic virus. Sugarcane cultivars that are infected with virus can be handle only by tissue culture. Tissue culture techniques providing healhty planting material within short time and a huge amount. Rapid in vitro sugarcane propagation methodology provides physio-logically uniform material and requires

no disinfestations suitable for production of disease-free seed canes of newly developed varieties is tissue culture as Mustafa and Khan (2012) reported.

Healthy planting material for sugarcane is high pre-requisite for optimum sugar-yield production because as reported by Comstock and Miller (2004) plants of susceptible cultivars to S*ugarcane Yellow Leaf Virus* (SCYLV) in commercial fields usually have an incidence of 85 % or higher in Florida. That is why they had big effort to obtain S*ugarcane Yellow Leaf Virus* (SCYLV)-free infected planlets through tissue culture. Other viruses which are found reducing sugar yield production is *Sugarcane Mosaic Virus* (SCMV). Based on review Sengar *et al.*, (2011) mosaic disease of sugarcane occurs throughout the world except few countries and was first reported from India.

Tissue culture also play great role for sugarcane breeding. Mostly, the pattern of plant breeding nowadays is genetic transformation. Transgenic sugarcane that resistance to herbicides and mozaic virus has assembled through genetic transformation. Sugarcane transformation has a high dependency on tissue culture, for example applying particle bombardment on embryogenic callus in order to get sugarcane resistant to herbicides (Gallo-Meagher *et al.*, 2000).

Since the selection of desirable traits would be done at callus stage, a part tissue culture stages. Selection must be done to determine which one is brings desirable traits and discard others no posses. Selection can be done either in field, greenhouse or in vitro. Mostly, workers considered to use in vitro selection rather than field ones. Since it brings a lot of advantages such as environmental factors under human control, allows the selection to a single factor and at the cellular level (Purnamaningsih *et al.*, 2013). Reliable callus proliferation and subsequent plant regeneration are important for selection of sugarcane in vitro (Sharma, 2005).

2.4 Callus

The term “callus” originates from Latin word *callum*, which means hard (Ikeuchi *et al.*, 2013). Callus defined as massive growth of cells and accumulation of callose because of wounding and biotic infection. In case of tissue culture,

callus is amorfis disorganized cell mass that growth around wounding point grown on nutrient media.

Initially, callus formation comes from cells within excised plants undergone dedifferentiation. Grafi (2004) defined dedifferentiation as commonly associated with reentry into the cell cycle, its distinguishing feature is the withdrawal from a given differentiated state into a 'stem cell'-like state that confers pluripotentiality. Eventhough cells within explants had differentiated, they still retain plasticity to dedifferentiate again and acquire new fates under genetic programme related to exogenous inducer such as nutrient, photopheriod, and temperature.

One of exogenous inducer is growing medium that should composed of nutrients and growth regulator. On one hand, nutrient provision promotes growth and on the other hand, growth generates 'demand' signals for nutrients. This feed-forward cycle is a key element of the integration of growth and nutrition and that it is built by dedicated signaling pathways partly involving hormones (Krouk *et al.*, 2011). Callus initiation in monocots require high concentration of auxin and low concentration of cytokinin while callus initiation in dicots require slightly higher of cytokinin than auxin (George *et al.*, 2008).

Commonly, callus can be divided into two kinds based on their morphogenetic, namely embryogenic and non embryogenic callus. The characteristic of embryogenic callus is yellow whitish or yellow cream, compact with nodular structure, and dried. Embryogenic ones is suitable for regeneration. Since embryogenic ones have globular bodies along with the firm consistency of the fragments (Jimenez and Fritz, 2001). These globular body will develop into leafy structure later on. Embryogenic callus is also determined by the availability of nitrogen sources that can be absorbed quickly by plants (Sukmadjaja and Mulyana, 2011). In opposite, non embryogenic callus can be recognized from its appearance such as friable, soft, translucent and watery. The growth rate of its callus is slow. Non embryogenic calli may fail to regenerate plants (Chakravarty and Goswami, 1999).

Callus age is a time span ranging from callus was formed until decline or death. The less age callus having a compact nodular nature, otherwise the long-term ones is friable (Jimenez and Fritz, 2001). Callus with less age have more totipotency as compared to old ones (Raja *et al.*, 1994). Callus growth seemed a case of sigmoid curves, where there is a rapid growth phase and continued to increase, and reached the peak of optimum and always continued to fall until eventually die (George *et al.*, 2008). In general, the rapid growth phase started since the first week upto the second months, with indication callus mass improvement due to differentiate cells around the wounding area. The ability of these cells differentiate will remain constant until passing the peak. The using of those kind of callus will show normal and easy-stimulated regeneration, when the ratio of hormones in the medium was changed to higher cytokinins than auxin. Passing the peak, callus growth will decline, its structure becomes more fragile and have a low regeneration ability. In shortly, callus age can play great role related with regeneration ability (Saad *et al.*, 2004).

Long term regenerable callus is the capability of callus to be able to regenerate even been cultured or stored for more than normally period. For sugarcane, callus are normally transferred into regeneration medium after two months. At that time, callus quite ready to enter regeneration phase. Effect of callus age on regeneration of sugarcane was ever reported by Anjum *et al.* (2012), Tiwari 2013, and Fiah (2014).

2.5 Regeneration

Regeneration is the creation of new form and organisation, where previously it was absent (George *et al.*, 2008). Regeneration pathways is classified into 2 kinds, somatic embryogenesis and organogenesis. Both of those pathways is required to rapid micropopagation. Motte *et al.* (2014) reported that plant regeneration in tissue culture is a critical step in most plant transformation and micropropagation procedures.

The performance of regeneration is affected by several factors such varied callus age, varieties and medium. The longest callus in culture medium, the worst

regeneration performences of its callus. It is proved by Tiwari *et al.* (2013) using two sugarcane varieties Cos 8820 and Cos 767 for in vitro regeneration. They showed that maximum organogenic response from morphogenic callus was noticed after 10 weeks of inoculation. By increasing the time of incubation for more than 13 weeks the rate of organogenesis was decreased and rate of necrosis was increased and even after 15 weeks 60 % callus became dead with very poor organogenic response.

The same pattern with previous results comes from four clones of Indonesia sugarcane named PS 862, PS 864, PS 881, and VMC 86-550 which has been proved by Fiah *et al.* (2014). Three months callus that experienced 3 times repeated subculture obtained from all clones has experienced a decline in the quality of callus and were not able to produce shoots anymore due to the lost of regeneration ability.

Regeneration ability is depend on genotype (Kamil *et al*., 2005). Anjum *et al.* (2012) showed that the best regeneration ability on sugarcane genotype S-2003-us-127 comes from 28 days old but on on S-2003-us-371 is different, the best one is 35 days old calli. That is strong evidence that genotype play great role to determine in vitro regeneration ability. Genetic basis to plant regeneration and cultivar specificity in regeneration response is a common problem in the tissue culture of several important crop plants (Pal *et al.*, 2011).

Formation of adventitious shoot and proliferation of axillary shoot in shoot cultures is optimized by high concentration of cytokinin while auxin is low concentration (George *et al.*, 2008). Cytokinins are the most efficient growth regulators that induce in vitro shoot regeneration. Genes involved in cytokinins metabolism or signal transduction are likely to affect shoot organogenesis (Meng *et al*., 2010). Cytokinins stimulate cell division and they release apical dominance effect of auxins, which can lead to the production of side-shoots (Scott, 2008). Cytokinins tend to pomote shoot proliferation from outer layer of callus. Recently, some researchers had already discovered *cytokinin related shoot induction gene*.

2.6 Hyphothesis

1. Callus characteristics is varied in various cultivars and callus ages.
2. There is interaction between callus age and sugarcane cultivar related to regeneration ability.

III. MATERIALS AND METHODS

3.1 Time and Place

The research was conducted from November 12th, 2013 to May 12th, 2014 at Center of Agricultural Biotechnology (CAB), Kasetsart University, Kamphaeng Saen campus No 1, Village 6, Malai Maen road, Kamphaeng Saen Sub-district, Kamphaeng Saen District, Nakhon Pathom 73140, Thailand.

3.2 Preparation

3.2.1 Equipment Preparation

Basic instrument alike laminair air flow cabinet was turned on and sterilized with UV radiation prior using. Culture bottles, petri dish, tweezers, and scalpel were washed and then sterilized by autoclaving at 121^0C for 20 minutes.

3.2.2 Media Preparation

There were two culture media used namely callus induction media and shoot regeneration media. Callus induction media was made from well-mixing of MS (Murashige and Skoog, 1962) basal medium, 20 g/l sucrose, 10% coconut water, and 3 mg/l 2,4-D. Shoot regeneration media was made from well-mixing of basic MS medium supplemented with 20 g/l sucrose and 10% coconut water. Both media were solidified with 7 g/l agar and pH adjusment of 5.7 prior to autoclave. The aliquot of 20 ml both medium was poured out into culture bottle.

3.2.3 Plant Material Preparation

Plant material in form of certain Thailand sugarcane (*Saccharum officinarum* L.) cultivars were obtained from Sugarcane Research Center, Kasetsart University at Kamphaeng Saen Campus, Thailand. Four high yielding local cultivars were chosen as plant materials namely LK 95-127, LK 92-17, K 93-219, and K 93-347. Sugarcane was 7 months-old at the time of used. Top shoot of canes were collected by using chopper knife. Top shoot of cane, composed of 2-4 alternating nodes and internodes, was further processed manually by using

chopper knife to produce smaller plant material in form of young leaf rolls with the lenght of 12 - 15 cm and 1 cm in diameter.

3.2.4 Explant Preparation

The surface sterilization was conducted in all plant material. Plant material were washed and were immersed in two concentration of 25% and 10% commercial bleach solution (Haiter™ ai: sodium hypoclorite), respectively. Each step took 20 minutes with continuously placed on 120 rpm shaker. Then, explants were subjected to three successive washings with sterile RO water. All washing protocol was done inside culture bootle and under laminary air flow cabinet. After sterilization, explant was transfered on petri dish and outer three whorls of explants was removed by using tweezers. The inner spindle was cut by using scalpel into approximately 20 mm pieces as explant.

3.3 Callus Induction and Plant Regeneration Ability of Various Callus Ages and of Certain Thai Sugarcane Cultivars

Experimental design used was completely randomized design (CRD) with two factors. The first factor was sugarcane cultivars (v) comprised of four levels, namely: LK 95-127, LK 92-17, K 93-219, and K 93-347. The second factor was callus ages (u) which consisted of three levels, namely two months, three months, and four months. All treatments replicated ten times. The experiment composed of two stages, callus induction and shoot regeneration. Accordingly, there were in a total of 3x4x10 = 120 experimental units each stage.

3.4 Procedure

3.4.1 Callus Induction

The explants pieces were placed onto callus induction medium (CIM) under laminair air flow cabinet as aseptic protocol. The cultures were incubated in multi shelf culture room with dark condition at regime 27 degree celcius. Callus induction stage run up to four months with monthly subcultured onto fresh CIM.

3.4.2 Shoot Regeneration

Calli were transplanted to shoot regeneration medium under laminair air flow cabinet as aseptic protocol. The cultures is placed under white florescent light with intensity 55 micromol/ m^2/secon, six hour/day and the room temperature adjusted to 7±2°C. All cultures were maintained within two months and transferred onto fresh medium (subculture) every month.

3.5 Response Variables

Observations were made since beginning on callus induction until shoot regeneration stages. All response variables was described as described below.

a. Percentage of embryogenic callus formed was counted at the end of callus induction stage prior to transplant onto regeneration medium. It was counted by following formula.

$$(\%)\ \text{embryogenic callus formed} = \frac{explant\ with\ embryogenic\ callus\ formed}{total\ explant}\ x\ 100\%$$

b. Physical appearance of callus such as colour, and structure were done by visually estimated at the end of callus induction stage prior to transplant onto regeneration medium.
c. The diameter of callus measured by connecting two farthest points of callus with ruler and showed in centimeter unit. It was counted at the end of callus induction stage prior to transplant onto regeneration medium.
d. The percentage of shoot regeneration was counted after two months of shoot regeneration stage. It was counted by following formula.

$$(\%)\ \text{shoot regeneration} = \frac{number\ of\ shooty\ calli\ with\ shoot\ greater\ than\ 1\ cm}{total\ \ number\ of\ calli}\ x\ 100\%$$

e. The lenght of shoot was counted by measuring shoots from the base of shoot up to the highest growing point and showed in centimeter unit. It was counted after two months of shoot regeneration stage.
f. The number of shoots was counted only for shoots greater than 1 cm of height. It was counted after two months of shoot regeneration stage.

3.6 Data Analysis

There were two kinds of data obtained, quantitative and qualitative data. Qualitative data consisted of colour and structure of callus. Qualitative data was shown in form of tables and photos. Quantitative data such percentage of embryogenic callus, callus diameter, percentage of shoot regeneration, the number of and lenght of shoot were tested using analysis of variance (ANOVA). The significant differences between treatments was manually analyzed by Duncan's Multiple Range Test (DMRT) with alpha (α) = 0.05.

IV. RESULTS AND DISCUSSION

The effect of different cultivars on percentage of embryogenic callus formed was significantly different (F calculated = 3.00, P = 0.03, α 5%). Percentage of embriogenic callus ranged from 83.90% in cultivar LK 92-17 to 100% in cultivar K 93-219. The effect of combination between various cultivars and callus ages was not significantly different (F calculated = 0.25, P = 0.96, α 5%). However, percentage of embryogenic callus ranged from 80% to 100%. The highest value of that variables was combination of K 93-219 with all level of callus ages and LK 95-127 or K 93-347 with two and three months while the lowest percentage of embryogenic callus was combination on LK 92-17 with three months callus.

Post host test using DMRT at α 5% revealed that K 93-219, LK 95-127 and K 93-347 were not significantly different each other in form of percentage of embryogenic callus formed. Three cultivars above produced more embryogenic calli than LK 92-17. LK 92-17 was significantly different to others and produced less percentage than others.

Embryogenic callus was indicated by compact with nodular structure, whitish yellow, and wide diameter (Figure 4.1 B). In other hand, non embryogenic ones was characterized by friable, translucent or black colour, and small diameter (Figure 4.1 A). Embryogenic callus was easier to regenerate than non embryogenic ones. Since embryogenic callus had nodular bulge as candidate of organ.

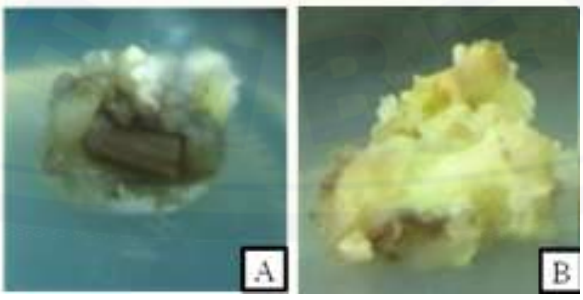

Figure 4.1 Callus of sugarcane : non-embryogenic (A) and embryogenic (B) callus

Structure of embryogenic callus was compact with nodular structure as depicted in Figure 4.2. This structure was not easily to broken because of compose of nodular cells with dense structure and sufficient water. Nodular body allowed the shoot coming after transferred onto shoot induction medium. So, it allegedly had a high resistance to the repeated subculture and medium with high auxin content like callus induction medium.

In opposite, friable callus was any form of fragile and crumb callus that compose of long tubular cells with tenuous ties between cells. Widyawati (2010) reported that the presence of endogenous hormones internally by the explants stymulate callus to be more friable. The longer maintenance in callus induction media (CIM), the more friable callus coming soon. However, this structure was high suitable for cell suspension culture because easy-handling and provide good aeration of oxygen between cells (Andaryani, 2010).

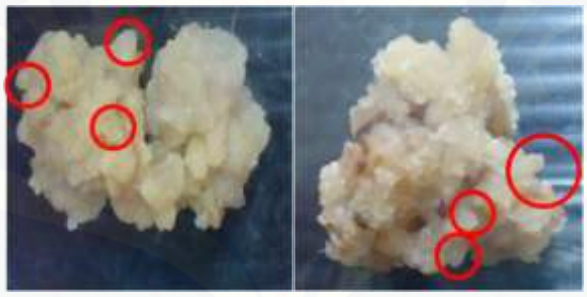

Figure 4.2 Structure of embryogenic callus with nodular bulge inside red circle

Colour of callus was one of physical appearance to figure out wheater callus still regenerable or not. Colour of callus varied such as whitish yellow, green, black and brown. In sugarcane, the colour of embryogenic callus was normally whitish yellow or yellow cream as depicted in Figure 4.3. The colour of whitish yellow was caused by the accumulation of flavonoid inside massive cell formed. In this experiments, most cultivars tested showed the similarity of whitish yellow except K 93-347 with additional brown spot at the bottom of callus due to more phenolic compound production. Browning was caused by oxidized phenolic compounds that result from polyphenol oxidase activity (Takahashi and Takamizo, 2013). Phenolic compounds was produced by callus in response to

wounding that occurred during subculture. The phenol compound used to protect the injured cells from invading pathogens. The excessive accumulation of phenolic compound were toxic and inactivates the growth of the tissue culture or even cause tissue death (Yusnita, 2004 ; Ahmad *et al*., 2013).

Figure 4.3 The colour of callus formed : A) whithis yellow and B) withish yellow with brown bottom

Callus diameter was an indicator of callus growth during the passage of time and also a determinant of regeneration. The diameter of callus was identical to the surface area. Surface area was comfort zone for the initiation of cell division. The outer layer of the callus provided oxygen, light and nutrients in higher amount. In addition, the outer layer allowed the release of CO_2 gas smoothly and rapidly degraded volatile inhibitor compounds. The availability of abundant amounts of exogenous and endogenous auxin was a key factor in the increase of diameter callus. The longer maintenance in callus induction medium allowed massive cell growth during exponential and linear phase, as consequently would make the diameter of the callus more widespread than previous time.

The interaction effect of combination between various cultivars and callus ages was significantly different on diameter of callus (F calculated = 6.43, P = 0.00, α 5%). Diameter of callus ranged from 1.2 to 4.4 cm. The widest diameter was produced by combination of K 93-347 with 4 months callus while the smallest diameter was produced by combination of LK 92-17 with two months callus. The effect of single factor of callus ages on diameter of callus also significantly different (F calculated = 507.13, P = 0.00, α 5%). The highest value of diameter was 3.6 cm that obtained from four months callus, whereas the lowest

value of diameter was 1.8 cm that obtained from two months callus. The impact of different cultivar on diameter of callus was significantly different (F calculated = 141.57, P = 0.00, α 5%). The widest diameter was 3.2 cm that obtained from K 93-347, whereas the smallest diameter was 1.9 cm that obtained from LK 92-17.

Post host test using DMRT at α 5% showed that combination of all cultivars with two months callus was significantly different and smaller than three months. Combination of all cultivars with three months callus also significantly different and smaller than four months. Statistically, K 93-347 produced the widest diameter combined with all callus ages. In opposite, interaction of LK 92-17 with all various callus ages produced the smallest callus diameter than others and was significantly different with other cultvars.

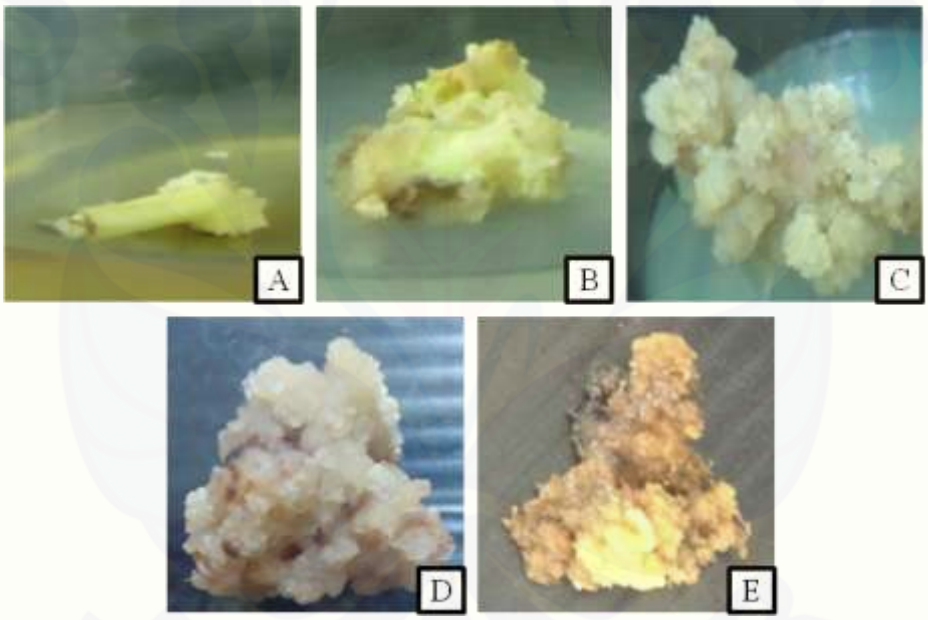

Figure 4.4 Callus development phase of sugarcane obtained present study where (A) callus initiation on wounded area of excised plant, (B) callus formation on wounded area and swelling of uninjured area, (C) exponential phase, (D) linear phase and (E) senescence phase

The changes of callus characteristics was reasonable since callus developed as the running of time. Callus developed to be similar with sigmoid curve where young cells carried out rapid growth until reach the peak and as getting mature, the growth might slows down until cell death (Figure 4.4). The

results successfully proved that all cultivars still showed any form of character that possible to support regeneration ability such high percentage of embryogenic callus, compact with nodular structure, whitish yellow, and wide diameter even after long term callus culture.

Response variable of percentage of shoot regeneration showed uniform data. All calli could be regenerated to be shoot greater than one cm within two months of regeneration stage. In other word, the percentage of shoot regeneration from all treatments was 100%. The effect of single cultivar either callus ages and cultivars was not significantly different on percentage of shoot regeneration. The effect of interaction between callus ages and cultivars on percentage of shoot regeneration also was not significantly different.

All cultivars used to date could survive and kept longer their competence state so that could be regenerated to form shoot after long term culture, specifically three and four months and also repeated subculture. Nevertheless, it did not mean no reduction of regeneration performances on each cultivars along with long term callus maintenance. The decline point clearly declared on response variabels of shoot lenght and shoot number.

The effect of single factor cultivars on the lenght of shoot was not significantly different (F calculated = 0.57, P = 0.64, α 5%). In other hand, single factor of callus ages showed the significant different effect on the same response variables (F calculated = 21.39, P = 0.00, α 5%). The highest value of shoot lenght was 14.3 cm that produced by two months callus while the lowest one was 8.93 cm that produced by three months callus. The effect of combination between different cultivars and callus ages on the lenght of shoot was significantly different (F calculated = 6.25, P = 0.00, α 5%). Shoot lenght ranged from 7.3 to 16.4 cm. The shortest shoot was produced by LK 95-127 with three months callus while the longest ones was obtained from combination of K 93-219 with two months callus.

Post host test using DMRT at α 5% showed that combination of K 93-219 with two months callus produced the longest shoot and was not significantly different with cultivar LK 95-127 and K 93-347 as the same callus age. In

opposite, the shortest shoot produced by combination of three months with K 93-219 and was not significantly different with combination of three months of LK 95-127 and K 93-347. Two months callus combined with LK 92-17 produced shorther shoot than others and was significantly different with K 93-219 and K 93-347. Three months callus combined with LK 92-17 produced longer shoot than others and was significantly different with most cultivar except K 93-347. Four months callus combined with LK 95-127 produced longer shoot than others and was significantly different with others cultivars.

The interaction effect of combination between different cultivars and callus ages on the number of shoot greater than 1 cm was significantly different (F calculated = 5.08, P = 0.00, α 5%). The number of shoot ranged from 10.2 to 34.2 shoots. The highest number of shoot was produced by combination of LK 95-127 with two months callus while the lowest one was produced by combination of K 93-219 with four months callus. The effect of single factor of callus ages on the number of shoot also significantly different (F calculated = 30.44, P = 0.00, α 5%). The highest number of shoot was 24.3 shoots that obtained from four months callus, whereas the lowest number of shoot was 13.9 shoots that obtained from two months callus. Single factor of cultivars showed the significant different effect on the same response variables (F calculated = 12.99, P = 0.00, α 5%). The lowest shoot number was 13.3 shoots that obtained from LK 92-17, while the highest shoot number than others was 21.7 shoots that derived from LK 95-127.

Response variable of shoot number tested by post host test using DMRT at α 5%. It showed that combination of LK 95-127 all with two months callus ages produced the highest number of shoot and was significantly different than others. In opposite, the lowest number of shoot produced by combination of four months callus with LK 92-17 and K 93-219. Two months callus combined with LK 95-127 produced the highest number of shoot and was significantly different compared to combination of the same ages with other cultivars. Three months callus combined with K 93-347 produced the highest number of shoot but it was not significantly different compared to combination of the same ages with LK 95-

127 and LK 92-17. Four months callus derived from K 93-347 produced the highest number of shoot but it was not significantly different compared to combination of the same ages with LK 95-127. The effect of callus ages on shoot number in cultivar K 93-347 and LK 92-17 was not significantly different. In opposite, the effect of callus ages on shoot number in cultivar LK 95-127 and K 93-129 was significantly different.

Various number of shoot regenerated was caused by the different amount of cell within callus in competent state. Cell in competent state was required for regeneration pathways through organogenesis. Not all cells able to stay in a state of competence, because naturally, the cells had the option to be competence or not depend on its original capacity, in the previous ground phase. The competence thus obtained was transient (Pulianmackal *et al*., 2014). Competent was a state of the cells that still maintain its capacity for a particular kind of cellular differentiation so that able to respond extra cellular signals. The competence to regenerate was common feature for many cell types at least in the meristematic zone. Explant used for present study is inner spinde, composed of apical meristematic tissues.

Furthermore, competent cell reacted to extra cellular signals and carried out cellular differentiation during determination state. It was proved by green bumps measured about two mm originally grew on callus surface. The genes encoding proteins targeted to chloroplasts were found in 2.2-fold excess over their frequency in the total genome, reflecting the fact that greening occurs (green callus formation) during these stages (Che *et al.*, 2006). The elongation of green bumps as encode by certain genetically program promoted the shoot arisen. Elongation of bumps to be green shoots run during the developmental phase as a continuation of the morphogenetic determination that has been fixed. Those process turned to irreversible and called canalized. Shoot primordia grew from nodular bulge with green colour in callus surface as induced by cytokinins to stimulate the formation of chlorophyll (Andaryani, 2010). The final product of canalized was shoot. The pathways of shoot organogenesis was described in Figure 4.5 below.

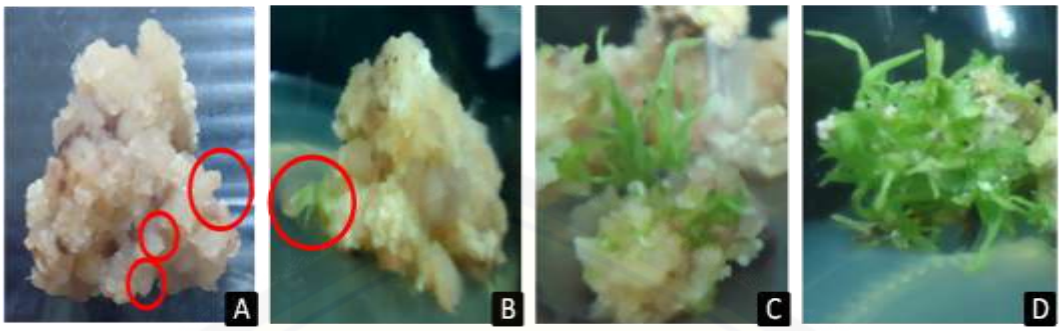

Figure 4.5 Shoot induction phase of sugarcane obtained present study where (A) nodular bulge in embryogenic callus, (B) emerging of green shoot primordia at the nodular place, (C) shoot coming up on outer layers of callus and (D) massive growth of shoot covering the callus.

Theoritically, as callus getting older the amount of competence cell within callus also getting lower and later on caused the decline of shoot number. Various callus ages used to date simply related to various performances of shoot number. The younger tissue within two months callus was proved to have more shoot than older callus such three and four months callus. It suggested that younger tissue have higher amount of competence state than old tissue.

Among various response, determination of long term regenerable cultivar was highly required to support mass-micropopagation through tissue culture, genetic transformation and virus-free plantlet production. Long term maintenance was refered to callus culture that longer than two months. Long term regenerable cultivar was cultivar that able to keep longer their competent state of callus after long term callus culture. The cells were able to perform particular kind of cellular differentiation later on after placed onto induction medium. Sugarcane cultivar was determined to be long term cultivars if only able to induce embryogenic callus regularly and regenerate normally even after long term maintenance culture. It could be maintain the number and length of shoots formed relatively stable in all various ages.

Present study showed that all cultivar potential to be long term in vitro regenerable cultivar. The evidence was 100% of shoot regeneration even after four months callus culture. It was different with results of previous researchers. Tiwari reported that 60% of 15 weeks calli death and cannot regenerate. Those

calli was derived from two sugarcane cultivars namely Cos 8820 and Cos 767. Fiah *et al.*, (2014) reported that 12 weeks culture and three times subcultured caused the death of callus tissues indicated by black tissue colour and lost of regeneration ability. Cultivar S-2003-us-371 was combined with 21, 28, 35 day old callus couldnot be regenerate anymore (Anjum *et al*, 2012).

Treated cultivar namely LK 95-127 was sugarcane cultivar with the highest number of shoots only at combination with two months callus. Unfortunately, the regeneration ability of the callus had fallen as callus getting mature. K 93-219 cultivars had the same properties as previous cultivar that the number of and lenght of shoots sharply declined as consequent of aging. LK 92-17 was the worst cultivars compared to others. The performances of regeneration in form of shoot number was lowerr than others. The last cultivar, K 93-347 was highly potential to be long term regenerable cultivar. Since it had good and stable regeneration performances indicated by 1) high embryogenic callus and fully regenerated shoot after long term maintenance culture, 2) more stable pattern in terms of number and length of shoots formed at each age level, 3) sligthly decline of regeneration performances. The decline as consequently of aging was not significantly different in variable of shoot number formed. In short, K 93-347 was long term in vitro regenerable cultivar.

Various response of cultivars in terms of shoot lenght and shoot number was also caused by the differences of genotype. Each sugacane cultivar had their own genotype that was contributed to regeneration ability. In vitro regeneration was linked to the performance of several genes that responsible for shoot induction. This study had not include comparison of genotypes or any molecular level test yet. The challenge of sugarcane sequencing project was the size around 10 Gb and complexity of its genome structure that highly polyploid and aneuploid with a complete set of homologous genes predicted to range from eight to ten copies of alleles (Cunff, *et al.*, 2008). Future work was expected to reveal genes responsible for optimizing the number and lenght of shoot produced by in vitro propagation in sugarcane.

V. CONCLUSION

5.1 Conclusion

Present reseach concluded several point as stated below.

1. Most of treated cultivars produced 100% of embryogenic calli, except LK 92-17. Embryogenic callus characteristics were whitish yellow, compact with nodular structure. K 93-347 produced the widest diameter calli than others.
2. All treated calli could be regenerated into shoot after long-term maintenance (four months) on callus induction medium with high auxin and repeated subculture. Interaction of two months callus and LK 95-127 produced the highest number of shoot than others. Combination of two months callus and K 93-347 and K 93-219 produced the longest shoot than others.
3. Long term in vitro regenerable cultivar was K 93-347.

5.2 Recommendation

Further research related to genes controlling the number of and lenght of shoot is needed to reveal the effect of various genotype on in vitro regeneration of sugarcane.

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APPENDIX

Table 1. Murashige and Skoog (MS) basal medium formula used for callus induction and shoot regeneration

No.	Stock	MS (mg/l)	10x (g/l)	100x (g/l)	200x (g/l)
1.	**Stock Solution I**				
	NH4NO3	1650	16.5	-	-
	KNO3	1900	19	-	-
	CaCl2.2H20	440	4.4	-	-
	KH2PO4	170	1.7	-	-
	MgSO4.7H2O	370	3.7	-	-
2.	**Stock Solution II**				
	H3Bo3	6.2	-	-	1.24
	Na2MoO4.2H2O	0.25	-	-	0.05
	CoCl2.6H2O	0.025	-	-	0.005
	KI	0.83	-	-	0.166
	MnSO4.H2O	22.3	-	-	4.46
	ZnSO4.H2O	10.93	-	-	2.186
	CuSO4.5H2O	0.025	-	-	0.005
3.	**Stock Solution III**				
	Disodium EDTA	37.25	-	3.725	-
	FeSO4.7H2O	27.85	-	2.785	-
4.	**Stock Solution IV**				
	Thiamin-HCl	0.1	-	0.2	-
	Pyridoxine-HCl	0.5	-	0.05	-
	Nicotinic acid	0.5	-	0.05	-
	Glycine	2.0	-	0.01	-
	Myo-Inositol	100	-	10.0	-

Table 2. Percentage of embryogenic callus formed from various callus ages and cultivars of sugarcane

Cultivar	Calllus Age	Repetition										Mean	Total
		1	2	3	4	5	6	7	8	9	10		
LK 95-127	2 months	10	10	10	10	10	10	0	10	10	10	9.00	90
	3 months	10	10	10	10	10	10	10	10	10	10	10.00	100
	4 months	10	10	10	10	10	10	10	10	10	10	10.00	100
LK 92-17	2 months	10	0	10	0	10	10	10	10	10	10	8.00	80
	3 months	10	10	0	10	10	0	10	10	10	10	8.00	80
	4 months	10	10	10	10	10	10	0	10	10	10	9.00	90
K 93-219	2 months	10	10	10	10	10	10	10	10	10	10	10.00	100
	3 months	10	10	10	10	10	10	10	10	10	10	10.00	100
	4 months	10	10	10	10	10	10	10	10	10	10	10.00	100
K 93-347	2 months	10	0	10	10	10	10	10	10	10	10	9.00	90
	3 months	10	10	10	10	10	10	10	10	10	10	10.00	100
	4 months	10	10	10	10	10	10	10	10	10	10	10.00	100
Total		120	100	110	110	120	110	100	120	120	120		1130
Mean		10	8.33	9.17	9.17	10	9.17	8.33	10	10	10	9.42	94.167

Table 3. The diameter of callus (cm) from various callus ages and cultivars of sugarcane

Cultivar	Calllus Age	Repetition										Mean	Total
		1	2	3	4	5	6	7	8	9	10		
LK 95-127	2 months	2	2.2	2.2	2.1	2	2.3	1.8	2	1.6	1.8	2	20
	3 months	2.3	2.5	2.8	2.5	2.8	2.5	3.5	3.2	3.1	2.8	2.8	28
	4 months	3.6	3.4	3.5	3.2	3.2	3.6	4	3.8	3.4	3.3	3.5	35
LK 92-17	2 months	1	1.4	1.4	1.2	1.3	1	1.1	1.1	1.3	1.2	1.2	12
	3 months	1.3	1.6	2	1.5	1.8	1.7	1.9	1.9	2.5	1.8	1.8	18
	4 months	3	2.9	2.5	2.5	3	3.1	2.8	2.8	2.7	2.7	2.8	28
K 93-219	2 months	2	2.2	1.8	1.7	2	1.8	1.5	2	2.1	1.9	1.9	19
	3 months	3	2.9	3.3	2.8	2.7	3.4	2.5	2.9	3	3.5	3	30
	4 months	3.6	3.3	3.7	3.6	3.9	3.7	3.6	3.9	4	3.7	3.7	37
K 93-347	2 months	2	2.1	2.2	2	2.3	2.4	2	2.3	1.9	1.8	2.1	21
	3 months	3	3.2	3.4	3.5	3.6	3.3	3.2	3.1	2.9	2.8	3.2	32
	4 months	4.4	4.5	4	4.7	4.6	4.4	4.6	4	4.3	4.5	4.4	44
Total		31.2	32.2	32.8	31.3	33.2	33.2	32.5	33	32.8	31.8		324
Mean		2.6	2.6833	2.7333	2.6083	2.7667	2.7667	2.7083	2.75	2.7333	2.65	2.7	27

Table 4. Percentage of shoot regeneration from various callus ages and cultivars of sugarcane

Cultivar	Calllus Age	Repetition										Mean	Total
		1	2	3	4	5	6	7	8	9	10		
LK 95-127	2 months	10	10	10	10	10	10	10	10	10	10	10.00	100
	3 months	10	10	10	10	10	10	10	10	10	10	10.00	100
	4 months	10	10	10	10	10	10	10	10	10	10	10.00	100
LK 92-17	2 months	10	10	10	10	10	10	10	10	10	10	10.00	100
	3 months	10	10	10	10	10	10	10	10	10	10	10.00	100
	4 months	10	10	10	10	10	10	10	10	10	10	10.00	100
K 93-219	2 months	10	10	10	10	10	10	10	10	10	10	10.00	100
	3 months	10	10	10	10	10	10	10	10	10	10	10.00	100
	4 months	10	10	10	10	10	10	10	10	10	10	10.00	100
K 93-347	2 months	10	10	10	10	10	10	10	10	10	10	10.00	100
	3 months	10	10	10	10	10	10	10	10	10	10	10.00	100
	4 months	10	10	10	10	10	10	10	10	10	10	10.00	100
Total		120	120	120	120	120	120	120	120	120	120		1200
Mean		10	10	10	10	10	10	10	10	10	10	10.00	100

Table 5. The lenght of shoot regenerated from various callus ages and cultivars of sugarcane

Cultivar	Calllus Age	Repetition										Mean	Total
		1	2	3	4	5	6	7	8	9	10		
LK 95-127	2 months	20	9	11	12	20	17	12	17	11	17	14.60	146
	3 months	9	4.8	1.5	14.5	12	4.5	1.5	7.7	16	1.5	7.30	73
	4 months	13	13	13	11	17	11	12	15	12	25	14.20	142
LK 92-17	2 months	12	16	4.3	12.5	10	12	14	7.5	9.5	11	10.88	108.8
	3 months	10	13	8.5	9	18	14	14	11.5	10	12	12.00	120
	4 months	6	15	9	15	8	13	9	8	10.5	8	10.15	101.5
K 93-219	2 months	10	20	12	22	12	20	21	17	14	16	16.40	164
	3 months	6	10	4	11	11	5	4.5	1	5.3	10	6.78	67.8
	4 months	11	9.5	5	5.5	4.7	7.5	11.5	10.5	16	18.5	9.97	99.7
K 93-347	2 months	12	14	12	13	15.5	22	14	19	18	14	15.35	153.5
	3 months	7	14	14.5	7.5	13	7.5	11	11	5.5	5.5	9.65	96.5
	4 months	9	5	9	5	10.5	9.5	8.5	6	7.5	8	7.80	78
Total		125	143.3	103.8	138	151.7	143	133	131.2	135.3	146.5		1350.8
Mean		10.417	11.942	8.65	11.5	12.642	11.917	11.083	10.933	11.275	12.208	11.26	112.567

Table 6. The number of shoot regenerated from various callus ages and cultivars of sugarcane

Cultivar	Calllus Age	Repetition										Mean	Total
		1	2	3	4	5	6	7	8	9	10		
LK 95-127	2 months	28	36	31	39	33	37	36	33	28	41	34.20	342
	3 months	16	13	5	16	12	25	9	19	23	20	15.80	158
	4 months	15	10	10	14	11	13	18	21	25	14	15.10	151
LK 92-17	2 months	18	11	10	15	8	21	9	16	27	11	14.60	146
	3 months	10	17	19	20	14	14	15	24	8	7	14.80	148
	4 months	5	12	14	14	8	6	12	14	9	11	10.50	105
K 93-219	2 months	18	27	21	17	20	18	21	24	38	36	24.00	240
	3 months	23	8	16	23	9	8	12	1	5	21	12.60	126
	4 months	10	9	3	10	10	8	13	3	18	18	10.20	102
K 93-347	2 months	21	17	18	27	29	19	29	30	33	20	24.30	243
	3 months	20	27	18	17	17	17	17	25	30	12	20.00	200
	4 months	41	19	22	12	17	20	34	15	11	5	19.60	196
Total		225	206	187	224	188	206	225	225	255	216		2157
Mean		18.75	17.167	15.583	18.667	15.667	17.167	18.75	18.75	21.25	18	17.98	179.75

Table 7. Analysis of variance of percentage of embryogenic callus formed

Source of variance	DF	SS	MS	F's counted		F table 5%	F table 1 %
Treatment	11	69.17	6.29	1.15	ns	1.886	2.432
Cultivar (v)	3	49.17	16.39	3	*	2.696	3.896
Callus age (u)	2	11.67	5.83	1.07	ns	3.086	4.828
v*u	6	8.33	1.39	0.25	ns	2.194	2.992
Error	108	590	5.46				
Total	119						

Table 8. Analysis of variance of diameter of callus

Source of variance	DF	SS	MS	F's counted		F table 5%	F table 1 %
Treatment	11	94.4	8.58	134.32	**	1.886	2.432
Cultivar (v)	3	27.13	9.04	141.57	**	2.696	3.896
Callus age (u)	2	64.8	32.4	507.13	**	3.086	4.828
v*u	6	2.47	0.41	6.43	**	2.194	2.992
Error	108	6.9	0.06				
Total	119						

Table 9. Analysis of variance of the lenght of shoot

Source of variance	DF	SS	MS	F's counted		F table 5%	F table 1 %
Treatment	11	1168.51	106.22	7.45	**	1.886	2.432
Cultivar (v)	3	24.34	8.11	0.57	ns	2.696	3.896
Callus age (u)	2	609.5	304.75	21.39	**	3.086	4.828
v*u	6	534.67	89.11	6.25	**	2.194	2.992
Error	108	1538.99	14.25				
Total	119						

Table 10. Analysis of variance of the number of shoot

Source of variance	DF	SS	MS	F's counted		F table 5%	F table 1 %
Treatment	11	5259.83	478.17	11.85	**	1.886	2.432
Cultivar (v)	3	1572.83	524.28	12.99	**	2.696	3.896
Callus age (u)	2	2457.45	1228.73	30.44	**	3.086	4.828
v*u	6	1229.55	204.93	5.08	**	2.194	2.992
Error	108	4359.1	40.362				
Total	119						

Table 11. Post-hocs test of effect of single factor of vaious cultivars to the percentage of embryogenic callus formed

Cultivar (v)	Callus age (u)			Mean
	2 months (u1)	3 months (u2)	4 months (u3)	
LK 95-127 (v1)	90	100	100	96.7 a
LK 92-17 (v2)	80	80	90	83.9 b
K 93-219 (v3)	100	100	100	100.0 a
K 93-347 (v4)	90	100	100	96.7 a

Note : Means followed by the same letter isnot significantly different, whereas means followed by different letter is significantly different based on DMRT at 5% significant level

Table 12. Post-hocs test of interaction various callus ages and cultivars to the diameter of callus

Cultivar (v)	Callus ages (u)		
	2 months (u1)	3 months (u2)	4 months (u3)
LK 95-127 (v1)	2.0_{a}^{C}	2.8_{b}^{B}	3.5_{b}^{A}
LK 92-17 (v2)	1.2_{b}^{C}	1.8_{c}^{B}	2.8_{c}^{A}
K 93-219 (v3)	1.9_{a}^{C}	3.0_{ab}^{B}	3.7_{b}^{A}
K 93-347 (v4)	2.1_{a}^{C}	3.2_{a}^{B}	4.4_{a}^{A}

Note : Means followed by the same letter isnot significantly different, whereas means followed by different letter is significantly different based on DMRT at 5% significant level

Table 13. Post-hocs test of interaction various callus ages and cultivars to the lenght of shoot

Cultivar (v)	Callus ages (u)		
	2 months (u1)	3 months (u2)	4 months (u3)
LK 95-127 (v1)	$14.6_{ab}{}^{A}$	$7.3_{b}{}^{B}$	$14.2_{a}{}^{A}$
LK 92-17 (v2)	$10.88_{b}{}^{A}$	$12_{a}{}^{A}$	$10.15_{b}{}^{A}$
K 93-219 (v3)	$16.4_{a}{}^{A}$	$6.78_{b}{}^{B}$	$9.97_{b}{}^{B}$
K 93-347 (v4)	$15.35_{a}{}^{A}$	$9.65_{ab}{}^{B}$	$7.8_{b}{}^{B}$

Note : Means followed by the same letter isnot significantly different, whereas means followed by different letter is significantly different based on DMRT at 5% significant level

Table 14 Post-hocs test of interaction various callus ages and cultivars to the number of shoot

Cultivar (v)	Callus ages (u)		
	2 months (u1)	3 months (u2)	4 months (u3)
LK 95-127 (v1)	$34.2_{a}{}^{A}$	$15.8_{ab}{}^{B}$	$15.1_{ab}{}^{B}$
LK 92-17 (v2)	$14.6_{c}{}^{A}$	$14.8_{ab}{}^{A}$	$10.5_{b}{}^{A}$
K 93-219 (v3)	$24_{b}{}^{A}$	$12.6_{b}{}^{B}$	$10.2_{b}{}^{B}$
K 93-347 (v4)	$24.3_{b}{}^{A}$	$20_{a}{}^{A}$	$19.6_{a}{}^{A}$

Note : Means followed by the same letter isnot significantly different, whereas means followed by different letter is significantly different based on DMRT at 5% significant level