INDUKSI KALUS DAN KEMAMPUAN PEMBENTUKAN ANAKAN DARI EMPAT KULTIVAR TEBU LOKAL THAILAND (*Saccharum officinarum* L.) SECARA *IN VITRO*

CALLUS INDUCTION AND TILLERING CAPABILITY OF FOUR THAILAND SUGARCANE CULTIVARS (*Saccharum officinarum* L.) BY *IN VITRO*

SKRIPSI

Oleh

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PROGRAM STUDI AGROTEKNOLOGI
FAKULTAS PERTANIAN
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Diajukan guna melengkapi tugas akhir dan memenuhi salah satu syarat untuk menyelesaikan Program Sarjana (S1) pada Program Studi Agroteknologi Fakultas Pertnian Universitas Jember

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PERSEMBAHAN

Dengan memanjatkan puji syukur kehadirat Tuhan Yang Maha Agung, skripsi ini saya persembahkan untuk :

1. Alm. Setjo Soetanto dan Alm. Cuk Soerodjo sebagai sumber inspirasi sejak kecil,
2. Keluarga besar,
3. Semua guru dan pendidik semasa hidup,
4. Teman hidup dan semua orang yang haus akan ilmu dan kebajikan,
5. Desi Hendriyani sebagai tempat berbagi hati,
6. Almamater Fakultas Pertanian Universitas Jember.

MOTTO

"It is better to travel well than to arrive"

(Sidharta Gautama)

"Carilah bunga kehidupan. Jadilah insan yang memahami hidupan,bukan insan yang menggurui hidup."

(Ayahanda Drs. Setyo Heru Budianto, S.E.)

SURAT PERNYATAAN

Saya yang bertanda tangan dibawah ini :

Nama : Arghya Narendra Dianastya

NIM : 111510501105

Menyatakan dengan sesungguhnya bahwa karya tulis ilmiah yang berjudul: **"Induksi Kalus dan Kemampuan Pembentukan Anakan dari Empat Kultivar Tebu Lokal Thailand (*Saccharum officinarum* L.) secara *in vitro*"** adalah benar hasil karya sendiri, kecuali disebutkan sumbernya dan belum pernah diajukan pada institusi manapun, serta bukan karya jiplakan. 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 mendapatkan sanksi akademik jika ternyata di kemudian hari pernyataan ini tidak benar.

Jember, 9 Oktober 2015

Yang menyatakan,

Arghya Narendra Dianastya
NIM 111510501105

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Tittle **Callus Induction and Tillering Capability of Four Sugarcane Cultivars (*Saccharum officinarum* L.) under *in vitro* Culture**

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RINGKASAN

Induksi Kalus dan Kemampuan Pembentukan Anakan dari Empat Kultivar Tebu Lokal Thailand (*Saccharum officinarum* L.) secara *in vitro*; Arghya Narendra Dianastya; 111510501105; Program Studi Agroteknologi; Fakultas Pertanian; Universitas Jember.

Tebu (*Saccharum* spp hybrid) adalah tanaman tahunan dengan poliploid tinggi yang termasuk dalam keluarga Poaceae dan suku Andropogoneae. Perkembangbiakan secara *in vitro* dibutuhkan untuk mencukupi permintaan tebu yang terus meningkat, dikarenakan pendekatan bioteknologi menawarkan keuntungan seperti multiplikasi yang cepat dan menghasilkan tanaman yang bebas penyakit. Tujuan dari penelitian ini adalah untuk mengetahui kemampuan induksi kalus, regenerasi tunas, dan pertumbuhan anakan dari 4 kultivar tebu lokal Thailand K92-80, KK3, LK95-127, dan K93-219. Media induksi kalus adalah MS + 2,4-D 3.0 mg/L + sukrosa 2% + air kelapa 10% (V/V) + agar 0.7%. Media yang digunakan untuk regenerasi tunas dan pertumbuhan anakan adalah MS + air kelapa 10% (V/V) + sukrosa 2% + agar 0.7%. Pada tahap induksi kalus, K92-80, KK3 dan K93-219 memiliki prosentase induksi kalus tertinggi (100%). Kultivar terbaik pada regenerasi tunas adalah LK95-127 yaitu 72.72% pada prosentase pertumbuhan tunas dan 5.27 pada rata-rata jumlah tunas yang dihasilkan. Kultivar terbaik pada tahap kemampuan anakan adalah KK3 yang memiliki 4.54 untuk rata-rata jumlah anakan yang dihasilkan. Faktor endogen memiliki peran tinggi pada induksi kalus, regenerasi tunas dan kemampuan anakan dari 4 kultivar tebu dengan kultur *in vitro*.

SUMMARY

Callus Induction and Tillering Capability of Four Sugarcane Cultivars (*Saccharum officinarum* L.) by *in vitro*; Arghya Narendra Dianastya; 111510501105; Study Program of Agrotechnology; Faculty of Agriculture; Jember University.

Sugarcane (*Saccahrum* spp. hybrid) is high polyploid parennial grass belong to the family Poaceae and tribe Andropogoneae. Propagation by *in vitro* is needed to fulfill the growing demand of sugarcane, since it offers advantages for rapid multiplication of cultivars and produces a healthy and disease-free plants. The objectives of this study was to know the capability of callus induction, shoot regeneration and tiller growth of 4 Thai local sugarcane cultivars (K92-80, KK3, LK95-127, and K93-219). The callus induction medium was MS + 3.0 mg/L of 2,4-D + 2% of sucrose + 10% (V/V) of CW + 0.7 % of agar. Medium for shoot regeneration and tiller production was MS + 10% (V/V) of CW + 2% of sucrose + 0.7% agar. In callus induction stage, K92-80, KK3 and K93-219 had the highest callus induction percentage (100%). The best cultivar in shoot regeneration stage was LK95-127 which had 72.72% in shoot regeneration percentage and 5.27 on the average number of shoots produced. The best cultivar in tillering capability stage was KK3 which had 4.54 on the average number of tillers produced. Endogenic factors is highly responsible in the callus induction, shoot regeneration and tillering capability of 4 sugarcane cultivars by *in vitro*.

PRAKATA

Puji syukur kehadirat Tuhan penguasa Semesta Alam, atas ijinnya penulis dapat menyelesaikan skripsi berjudul **Induksi Kalus dan Kemampuan Pembentukan Anakan dari Empat Kultivar Tebu Lokal Thailand (*Saccharum officinarum* L.) secara *in vitro*".** Skripsi ini diajukan guna memenuhi salah satu syarat untuk menyelesaikan Program Sarjana (S1) Pertanian pada Program Studi Agroteknologi, Fakultas Pertanian, Universitas Jember.

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I. INTRODUCTION

1.1 Background

Sugarcane (*Saccharum* spp. hybrids, family Poaceae, tribe Andropogoneae) is a high polyploid (*2n* = 36-170) perennial grass (Gallo-Meagher *et al*., 2000). It has ability to store high concentration of sucrose in the stalk and grows relatively rapid and produces high yields (Singh, 2010). It is commonly known that sugarcane is one of the most efficient photosynthesizer, C-4 plant in plant kingdom (Yadav and Ahmad, 2013). Commercial sugarcane today is mainly derived from the hybridization of the noble cane (*Saccharum officinarum*) with the cultivated species such as *S. sinese* and *S. barberi* or sometimes is the result of hybridization process of *S. officinarum* with the other two wild species which are *S. spontaneum* and *S. robustum* (Peng, 1984).

Sugarcane is cultivated in 127 countries in both the tropics and subtropics and covering an area up to 25.4 million hectares worldwide with a production of 1.79 billion tons in 2011, providing approximately 70% of the world's sugar supply. (Singh, 2010; Joshi *et al.*, 2013). The top 5 largest exporters are Brazil, Thailand, European Union, Australia, and Cuba. As number one exporter since 1985, Brazil has exported ten-fold, to over 10 million tonnes in 2003 and control the world sugarcane price (Kole, 2007). Thailand as one of the largest producer of sugarcane also increases the number of sugarcane production up to 99.5 million metric tons in 2012 (Prasertsri, 2013). According to the Departement of Agriculture (2001), Thailand become the biggest sugarcane exporter in 1998/99 when the sugarcane production was about 50 milion tonnes with an average yield of 55 tons/ha. The exported quantity at that time was approximately 2.6-3.9 million tonnes.

Sugarcane (*Saccharum officinarum* L.) is one of the most important cash and industrial crop and is widely cultivated for white refined sugar (Khamrit *et al.*, 2012). Nowadays, sugarcane is also used for ethanol due to its inexpensiveness, abundant and can be planted in vast region of the world. In 2013, approximately 104 million m^3 of ethanol produced worldwide, and approximately

50% of production was from sugarcane crops (Singh, 2010; CropEnergies, 2014). Besides that, sugarcane also produces valuable products such as biofibres, waxes, and bioplastic (Singh *et al.*, 2013).

Sugarcane breeding programmes have focused on the production of cultivars with high yield, higher sucrose content, pest and disease resistance, tolerance to abiotic stress and improved rooting ability (Yadav and Ahmad, 2013). However, improvement of sugarcane cultivar via conventional breeding is relatively slow due to the large and variable in genome size, complex ploidy levels, narrow genetic base, limited gene pool, and meiotic instability (Joshi *et al.*, 2013). The other problems of conventional breeding of sugarcane are lack of rapid multiplication due to multiplication rate through sett by conventional propagation is 1:8 (Abbas *et al.*, 2013) and continuous contaminations by systemic diseases (Visessuwan *et al.*, 1999). Consequently, breeding for superior traits is a difficult and taking 10-14 years to realease (Snyman *et al.*, 2010).

It has been realized that the growing demand of newly released sugarcane cultivars could not be fulfilled by only use the conventional methods of plant multiplication (Sengar *et al*., 2011; Yadav and Ahmad, 2013). Using new technology such as biotechnology offers excellent opportunities to improve sugarcane crop for specific targeted objectives such as high productivity and disease resistance in the short period of time (Sengar *et al.*, 2011). There are several areas of biotechnology research in sugarcane improvement today including: (1) cell and tissue culture techniques for molecular breeding and propagation; (2) engineering novel genes into commercial cultivars; (3) molecular diagnostics for sugarcane pathogens to improve exchange of *Saccharum* germplasm (Lakshaman *et al*, 2005).

Understanding tissue culture technique becomes the basic tools to conduct plant propagation via biotechnology (Neumann *et al.*, 2009). According to Hartmann *et al.* (1990), tissue culture can be defined as an aseptic culture of a wide range of excised plant parts. Plant tissue culture offers advantages over conventional methods of propagation for a large and rapid multiplication of cultivars with desirable traits and production of healthy and disease-free plants in

any season with conservation of space and time (Ahmadian *et al.*, 2013; Kataria *et al.*, 2013). Propagation by tissue culture also becomes an attractive and powerful tool in the research field throughout the world, especially in the area of large scale clonal propagation, crop improvement through genetic manipulation, conservation of plant genetic resources and valuable germplasm (Tarique *et al*., 2010).

As mentioned by Snyman *et al*. (2006), plant tissue culture of sugarcane offers the best methodology for quality and phytosanitary planting material at a faster rate in a shorter period of time as well as increases the propagation potential by 20-35 times. This is because regerenation through tissue culture can produce rapid production of sugarcane clones (Jabbott and Atkin, 1987). Sugarcane tissue culture also gives an advantage which effectively reduces the time period between selection and commercial release of new sugarcane cultivars (Abbas *et al*., 2013) as well as provides an alternatives control practice to overcome various kind of viruses and diseases (Visessuwan *et al.*, 1999).

Numerous studies on sugarcane plant regeneration have been reported. Successful culture and regeneration of plants from protoplasts, cells, callus and various tissue and organs have been achieved in sugarcane crops (Yadav and Ahmad, 2013). Attempt to measure callus growth and tillering capability by *in vitro* is particularly important. Understanding callus growth capability can be used as futher development of biotechnology in sugarcane, while understanding number of tiller by *in vitro* can be used as a guidance to understand the production yield. As it is mentioned by Yadaf (1991) that optimal number of millable canes dirrectly effect to the sugar and yield production. However, every sugarcane cultivar has different responses and variations from the treatment given. Attempt to conduct tissue culture experiment in different sugarcane cultivars is needed to find the disirable trait using short period of time. This research is conducuted to observe callus induction and tillering capability of 4 sugarcane cultivars using tissue culture technique. Sugarcane cultivars used are Thailand local cultivars : K92-80, KK3, LK95-127, and K93-219.

1.2 Objective

1. To know the callus induction capability of 4 Thai local sugarcane cultivars by *in vitro*.
2. To know the shoot regeneration via callus of 4 Thai local sugarcane cultivars by *in vitro*.
3. To know the tillering capability of 4 Thai local sugarcane cultivars by *in vitro*.

II. LITERATURE REVIEW

2.1 General aspects of *Saccharum officinarum* L.

Sugarcane belongs to the genus *Saccharum*, that firstly established by Linnaeus on Species Plantarum in 1753 with two species: *S. officinarum* and *S. spicalum*. The genus belongs to the tribe Andropogenae in the grass family, Poaceae. The tribe includes other tropical grass such as *Sorghum* and *Zea* (maize) (Kole, 2007).

The generic name *Saccharum* could be traced back to the Sanskrit word Karkara or Carkara, meaning gravel (Bakker, 1999). It symbolizes prosperity for it adorns the goddness of wealth, Sri Laxmi (Hunsigi, 1993). Records of sugarcane in history have been in existence since 510 BC where 'reeds which produce honey without bees' were first indicated by soldiers of the emperor Darius near the Indus river, India. However, The conquest of Alexander The Great of India in 327 BC made the sugar start to spread in the western world (Kole, 2007).

Modern sugarcane as we know it today evolved in 1893 with the successful crossing program between *S. officinarum* Black Chirebon (2n=80) and the wild *S. spontaneum* Kassoer (2n=40-128) (Kole, 2007; Joshi *et al*., 2013). According to Sengar *et al.* (2011), a series of backcrosses to *S. officinarum* resulted in cultivars with higher yields, improved ratooning ability and disease resistance in which Java breeder called this process as 'nobelization' (Babu, 1990). The process of "nobelization" of sugarcane as we know has resulted in a highly complex interspecific aneupolyploid genomic organization in sugarcane crops (2n=99–130). (Joshi *et al.*, 2013). Nowadays, over 400 clones of *S.officinarum* have been recorded. *S.officinarum* is generally characterized by having chromosome number of 2n=80, with basic chromosome number of x=10 (Kole, 2007). Most modern sugarcane breeding programs rely on extensive intercrossing of elite cultivars derived from these early hybrids (Lakhsaman *et al.*, 2005).

2.2 Classification of *Saccharum* L

Sugarcane belongs to the genus *Saccharum* L., traditionally placed in the tribe Andropogoneae of the grass family (Poaceae). This tribe includes tropical and subtropical grasses and the cereal genera *Sorghum* and *Zea* (known as maize or corn). The taxonomy and phylogeny of sugarcane is complicated and comes from five genera which share common characteristics and form a closely related interbreeding group known as the '*Saccharum* complex'. The *Saccharum* complex comprises *Saccharum*, *Erianthus* section *Ripidium*, *Miscanthus* section *Diandra*, *Narenga* and *Sclerostachya*. These genera are characterised by high levels of polyploidy (polyploids have more than two sets of chromosomes) and frequently unbalanced numbers of chromosomes (aneuploidy) (Kole, 2007).

2.3 Morphology of *Saccharum officinarum*

2.3.1 The root

The sugarcane root system is fibrous and shallow. There are two kinds of root of sugarcane. The first root is from primordial of the cutting, which are thin and branched, and the second root is from the primordial of the tillers that are thick, fleshy and much less branched. In the sugarcane, the top 25 cm of soil contains 50% of the plant roots, with the next 35 cm containing a further 40% of the roots. However, the effective root zone varies depending on the soil type (Peng, 1984).

2.3.2 The stem

Sugarcane has multiple stems or culms which height of mature sugarcane stem varies in the range of 3-5 meters and the diameter of stem varies in the range of 2-4 cm, depending on cultivars, internal and external growth factors. In every stem consists of a series of nodes separated by internodes. Each node consists of a growth ring or intercalary meristem. The node is the place where a leaf scar remain after the leaf has dropped (Peng, 1984). Internode length varies from each cultivar (Bakker, 1999). The basal region of internode, just above the leaf scar, is the root band (root ring) where the root primodia (root initials) are located. Below

the root band is the wax band, a zone covered with a layer of wax in varying density (Peng, 1984).

2.3.3 The leaf

The leaf of sugarcane consists of two parts, the blade and the sheath which separated by a leaf joint. The sheath which clasps the stem may be smooth or hairy. The leaves are attached alternately to the nodes. The basal leaves are very small but up to the stem, blades develop, gradually growing longer till they reach a maximum size. The leaf joint is located at the juction of the blade and the sheath. The number of green leaves increase as the plant grows older. During the boom phase of growth, the stalk of a healthy plant may contain as many as 17 – 20 leaves (Bakker, 1999).

2.3.4 The infloresence

The sugarcane inflorescence is an open branched panicle which also known as an arrow whose shape, degree of branching and size are highly cultivar specific. The arrow can bear thousands of flowers, and is estimated to average 24,600 florets. The arrow consists of a main axis and first, second and third order branches. Attached to the branches are spikelets arranged in pairs, one of which is sessile and one pedicellate, that bear individual flowers. At the base of each spikelet is a row of silky white hairs. Sugarcane flowers consist of three stamens as a male organ and a single carpel with a feathery stigma as a female organ. Sugarcane flower is a wind pollinated flowers. The male stamens may be abortive and reduced the pollen production (Australian Goverment, 2004).

2.4 Tillering

Tillering is characteristic of the grass family. In field propagation, tillering is defined as underground branching of sugarcane. Tillering is a phenomenon when the buds of a cutting start developing into shoots called mother shoots or primaries. The little stem of these primaries consists of many shoots which in turn may produce tertiary shoots. Tillering phase prevaileds only during the early growth stage. After an appropiate number of tillers are formed, each begins to undergo the elongation phase until maturity. Only a certain number of tillers will

successfully become millable stalks, due to competition for nutrients (Peng, 1984).

2.5 Sugarcane in Thailand

Sugar was known to the Thai people as sugar cake in the Sukhothai Dynasty (1219-1438 A.D.). The sugar producers during that time were cottage industries from Sukhothai, Phitsanulok and Kamphaeng Phet Province. In modern times, the sugar mill industry began in 1937 by the government. Lampang Sugar Mill was the first state enterprise sugar mill, followed by a second mill in Uttaradit in 1942 (Departement of Agriculture, 2001).

Sugarcane in Thailand grows best in deep, well drained loamy to loamy sand soil textures that have pH range between 6.1- 7.7 and an organic matter content not less than 1.5 %. In Thailand, clay textured soils are unfavorable to sugarcane growth. Optimal temperatures for growth are between 20 and 35^{o} Celcius. The water requirment is 1,200-1,600 mm/year (Departement of Agriculture, 2001).

There are many cultivars of sugarcane in Thailand. Cultivars should be chosen that are specifically adapted for that region. Cultivars such as K88-92, U Thong 3 and U Thong 1 are favorable because can be grown in almost every place in Thailand (Departement of Agriculture,2001).

2.5.1 Sugarcane cultivars

K92-80 is a non flowering cultivar as a result of hybrid cross between K84-200 and K76-4. K92-80 cultivar has a yield potential up to 118.8 ton/ha. K92-80 has fast growing capability with moderate tillering. In the case of ratooning, this cultivar has a very good ratooning and moderate drought stress tolerance.

KK3 is non flowering cultivar that very popular in Northeast region of Thailand which has sandy loam soil characteristic. KK3 is a progeny of 85-2-352 and K84-200. This cultivar has desirable traits such as fast growing, good ratooning, and high yield with average 113.1 ton/ha. One plant of KK3 can have

tiller up to 5 tiller per plant which is considered to be moderate tillering capability.

K93-219 is characterized by fast germination and growing with potential yield up to 125 ton/ha. This cultivar is non flowering cultivar as a result of hybrid cross between U-thong 1 and K84-200. Tillering capability of this variety is considered to be moderate with good ratooning capability. K93-219 also known as drought tolerant cultivar.

LK95-127 is a non flowering cultivar and also known as a cultivar that good for fresh juice cane. It is high yield cultivar with average of yield up to 112.5 ton/ha. This cultivar is good in ratooning with moderate tillering capability (4-5 stalks/plant).

2.6 Tissue culture

The concept of plant tissue and cell culture was mentioned in 1902 by the German botanist Gottlieb Harberlandt. Gottlieb Harberlandt published a paper entitled “Experiments on the culture of isolated cells”. Haberlandt had attempted to culture chlorophyll-containing cells and demonstrated the totipotency of cells. That experiment initiated a new method of plant propagation, which has known as 'Plant Tissue Culture' (Singh, 2003).

Tissue culture is a term used to indicate the aseptic culture (*in vitro*) of a wide range of excised plant parts. In many practice, propagators use the term micropropagation, *in vitro* and tissue culture interchangeably to mean any plant propagation using aseptic culture (Hartmann *et al.*, 1990). This definition also extends to the culture of excised embryos and protoplast culture. There are other terms have been used in micropropagation and tissue culture based on explant selection in relation to life cycle. These terms are meristem-tip culture, axilary shoot proliferation, adventitious shoot induction, organogenesis and somatic embryogenesis (Hartmann *et al*., 1990).

According to Hartmann *et al.* (1990), there are several important pathways of development of explant from tissue culture. The pathways are as follow:

1. Organogenesis may occur within the callus mass, to produce new plantlets.
2. Specific treatments may cause the cells to disassociate and develop a cell suspension culture.
3. Cells may be treated to produce a protoplast culture.
4. The regenerative potential may be shift toward somatic embryogenesis.

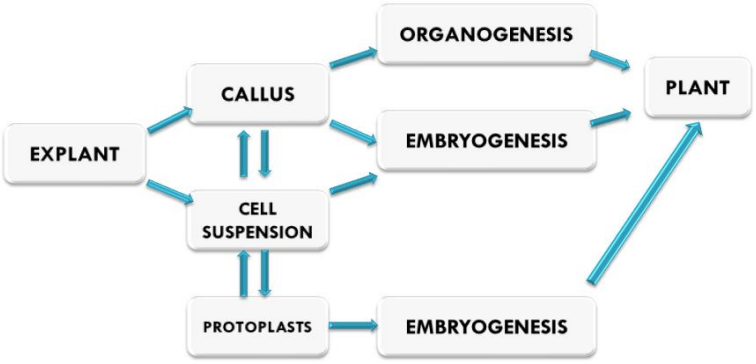

Figure 1: Regeneration pathways in plant tissue culture (Hartmann *et al*., 1990)

2.7 Totipotency

The basic concept of tissue culture is totipotency. Totipotency means an ability in individual plant cells to be regenerated to a whole plant by controlling culture conditions (Lee and Huang, 2013). In nature, totipotency can happen in the response of fast restoration of the lost or stress-damaged parts of shoots and roots. In *in vitro* conditions, practically any living cell with a nucleus can experience the process of dedifferentiation under the influence of nutrient medium components (Ezhova, 2003).

2.8 Micropropagation stages of tissue culture

Generally, according to Beyl and Trigiano (2008), there are 5 stages to establish micropropagation in plant, start to stage 0 to 4. Those stages are: (stage 0) donor plant selection and preparation, (stage I) establishment of aseptic culture, (stage II) proliferation of axillary shoot, (stage III) pretransplant or rooting, (stage IV) tranfer to natural environtment.

Stage 0 refers to selection and maintenance of the stock plants that used as the source of explants. Stock plants are maintained in clean and controlled environtment to avoid specific pathogens and unfavorable environtments. General objectives of Stage I are to place an explant into aseptic culture by avoiding contamination and provide an *in vitro* environment that promotes stable production. Contamination avoidance is conducted by sterilization. Sterilization is usually accomplished through surface disinfecting by alcohol or sodium hypochlorite to eradicate any kinds of bacteria, fungal and virus from plant. Most of Stage I media consist of mineral salts, sucrose, and vitamins, supplemented with plant growth regulator (PGR) (Beyl and Trigiano, 2008). Stage II is also known as Multiplication Stage has a purpose to mantain the explant in a stabilized state and multiply the microshoots to the number that suitable for rooting. Media used are slightly similiar with Stage I and commonly cytokinin is mainly used to shoot initiation process. Stage III has a function to produce root in explants and to prepare them for transplanting out of the aseptic protected environtment to the outdoor condition. Subculture is needed in this process and required an auxin hormone to induce root. The last stage is Stage IV in which the explant rooted are transplanted out side the culture vessel. In this stage, the microplants are transplanted into standard pasteurized rooting or soil mix in a small pots or cells in more or less conventional manner. Once the microplants are established in the rooting medium, the microplants should be gradually exposed to a lower relative humidity and higher light intensity (Hartmann *et al.*, 1997).

2.9 Organogenesis

Organogenesis begins with dedifferentiation of parenchyma cells to produce centers of meristematic activity called meristemoids. Dedifferentiation of parenchyma cells form a clumps of cell which also known as a callus (Hartmann *et al.*, 1997). Organogenesis involves the formation of organized structure like shoot and root from pre-existing structures such as unorganized mass of cells known as callus. Plant cultured through organogenesis can be achieved by two ways. The first is organogenesis through continuous development of callus formation with *de novo* origin also known as indirect pathway and second is from emergence of adventious organs such as lateral or axillary buds directly from the explant which also known as direct pathway (Chawla, 2003). Indirect regeneration often results in somaclonal variation making the strategy less desirable for large scale clonal multiplication. Therefore, direct regeneration without a callus phase is a reliable method for clone production (Kataria *et al.*, 2013).

2.10 Callus culture

Callus is an actively dividing non-organized mass of undifferentiated and differentiated cells often developing either from injury or wounding. In *in vitro*, callus is produced on explants from peripheral layers as a result of wounding and in response of growth regulators either endogenous or supplied in the medium. Callus provides an important tissue culture system because it can be subcultured and mantained more or less for an unlimited or unspecified period of time (Hartmann *et al.*, 1990). Explants from both mature and immature organs can be induced to form callus. However, explants with an active cells such as young and juvenil cells are generally good for callus initiation. Callus tissue form different plant species may be different in structure and growth habit. The callus growth differs among plant species. It depends on various factors such as the origin, position of the explant and the growth conditions (Chawla, 2003).

2.11 Component of *in vitro* media

The main components of most *in vitro* media are mineral salts and sugar as carbon source and water. Other components may include organic supplements, growth regulators and gelling agent.

2.11.1 Inorganic nutrients

The inorganic nutrients of a plant cell culture are those required by the normal plant. The inorganic nutrients consist of macronutrients and also micronutrients. Macronutrients are reqired in millimole quantities and micronunutrients are required in micromolar concentration (Thorpe, 1981). For most purposes a nutrient medium should contain at least 25 and up to 60 mM inorganic nitrogen. There are various ingredients of inorganic nutrient in *in vitro* for different stages of culture and plant species, but the basic MS (Murashige & Skoog) (Table 1) and LS (Linsmaier & Skoog) are most widely used (Kataria *et al.*, 2013). The Murashige and Skoog medium has been used widely for a range of culture types and species, particularly herbaceous plants and tissue culture. According to Hartmann and Kester (1983) This medium is rich in macroelements, particulary nitrogen, including nitrate (NO_3) and ammonium ions (NH_4) and vitamins.

Table 1 Murashige and Skoog medium composition.

Medium composition	**mg l^{-1}**
NH_4NO_3	1650.00
KNO_3	1900.00
$CaCl_2H_2O$	440.00
$MgSO_4.7H_2O$	370.00
KH_2PO_4	170.00
KI	0.83
H_3BO_3	6.20
$MnSO_4.4H_2O$	22.30
$ZnSO_4.7H_2O$	0.86
$Na_2MoO_4.2H_2O$	0.25

Table 1 (continue)

Medium composition	mg l^{-1}
$FeSO_4.5H_2O$	27.85
$Na_2EDTA.2H_2O$	37.25
Myo-inositol	100.00
Nicotinic acid	0.50
Pyridoxine-HCl	0.50
Thiamine-HCl	0.10
Glycine	2.00

2.11.2 Carbohydrate

During *in vitro*, carbohydrate plays an important role and act as an energy source required for growth, maintenance and differentiation of xylem and phloem element (Kataria *et al.*, 2013). Carbohydrate is also needed for inducing primary root and acts as an osmoticum and regulates the *in vitro* shoot proliferation. The most commonly used carbohydrate source is sucrose, but other sugar like glucose, fructose, dextrose, mannitol and sorbitol are also used. According to Lee and Huang (2013), explants uptake sucrose from the medium and hydrolyze it into glucose. Cell wall-bound invertase (CIN) and sucrose transporter (SUT) are the main routes for sucrose absorption and transportation in higher plants.

2.11.3 Plant growth regulator

Plant growth regulators (PGRs) have an important role in cell growth and differentiation. Both exogenous and endogenous levels of PGRs are highly related to shoot organogenesis (Lee and Huang, 2013). Among various growth regulators, auxins (NAA, IAA, IBA and 2,4-D), cytokinins (BAP, Kinetin, Zeatin), ABA, gibberellins and ethylene are very important. In *in vitro*, the nature of organogenic differentiation is determined by the relative concentration of auxins and cytokinins. Higher cytokinins to auxins ratio promotes shoot formation, while higher auxins to cytokinins ratio favours root differentiation (Kataria *et al.*, 2013).

a. Auxin

Auxin is synthesised by plant and it owes its name due to its effect on elongation of cells (auxesis). In *in vitro*, auxin plays an important role to induce cell multiplication and rhizogenic activity (Auge *et al.*, 1995). Indole-3-acetic acid (IAA) is the primary auxin in plants. IAA is a weak acid (pKa = 4.75) that is synthesized in the meristematic regions at the shoot apex and transported to the root tip in plants (Yong *et al.*, 2009). In sugarcane, callus is induced in the presence of auxin, either 2,4-D (2,4-dichlorophenoxy-acetic acid) or picloram (Ali *et al*., 2012). According to Nikolaeva *et. al.* (2008), 2,4-D promotes active proliferation of the cells and steady growth of callus and suspension cultures with the rate of callus formation depending on 2,4-D concentration and cultivar characteristics.

b. Cytokinin

Cytokinin is one of the plant hormones that crucial for plant growth and development and it is known to promote cell division and differentiation. Cytokinin can also stimulate lateral bud growth and cause multiple shoot formation by breaking shoot apical dominance (Jana *et al.*, 2013). Different concentration of cytokinin used affects the percentage of shoot regeneration, shoot numbers and shoot length (Bohidar *et al*., 2008).

The compounds of cytokinin include N^6-benzyladenine (BA), kinetin, N^6-isopentenyl-adenine (2iP) and zeatin (Hartmann *et al*., 1997). According to Ružić and Vujović (2008), cytokinins are classified into two major groups by their chemical structures which are synthetic phenylurea derivates and adenine derivates which may occur naturally. Zeatin and 2-isopentyladenine (2iP) are naturally occurring cytokinins, whereas, N^6 benzyladenine (BA), 6-Benzylaminopurine (BAP), 6-furfuryl-aminopurine (kinetin, Kin), and [1-Phenyl-3-(1,2,3,-thiadiazol-5-yl)] urea (thidiazuron, TDZ) are synthetic cytokinins (Jana *et al.*, 2013)

c. Coconut water

Coconut water is traditionally used as a growth supplement in plant *in vitro*. This is because there are many phytohormones in coconut water such as

auxin, cytokinin and gibberelline. The effect of coconut water on micropropagation was first discovered by Van Overbeek in 1942. The study focused the stimulatory effect of coconut water on the embryo development and callus formation in *Datura* and concluded that there are some complex substances in coconut water which are sometimes required in addition to growth hormones for callus induction and regeneration (Yong *et al.*, 2009).

Some of the most significant and useful components in coconut water in micropropagation are cytokinins, which are a class of phytohormones. Cytokinins can be found in young green coconut fruit. Coconut water contains various cytokinins such as kinetin and *trans*-zeatin. Kinetin is the first form of cytokinin discovered by human. It is a degradation product of herring sperm DNA and it is found to be able to promote cell division in plants. Kinetin is one of the cytokinins that has the effects on plant developmental processes that could be influenced by cytokinins, such as leaf expansion and seed germination. The other form of cytokinin found in coconut water is *trans*-zeatin. *Trans*-zeatin is the first naturally-occurring cytokinin identified from a plant source (*Zea mays*). *Trans*-zeatin is normally used to induce plantlet regeneration from callus in plant tissue culture (Yong *et al.*, 2009).

2.11.4 Agar

Agar is a powdered product obtained from certain species of red algae. Agar is used as a solidifying agent and assumed to be an neutral support for callus growth and multiplication (Kataria *et al.*, 2013). There are two factors that affect agar usage. Those two factors are concentration and pH (Hartmann and Kester, 1983). Normally, 0.8 percent agar is used for culture medium. A higher concentration of solidifying agent in the medium reduced vitrification, but in certain cases, an increase in amount of agar causes adverse effect (Kataria *et al.*, 2013). A pH of 5.0 to 6.0 is usually used. Acid or very low pH can cause deteroriation of agar and unproper solidify of agar (Hartmann and Kester, 1983).

2.12 Culture condition

Light is an important factor for the success of an *in vitro* experiment. The intensity, quality and extent of daily exposure of light are the determining factors in the *in vitro*. Cultures are usually maintained in a photoperiod of 16 hours of light photon flux density of 60 μmol m^{-2} s^{-1} and 8 hours of darkness. Temperature for *in vitro* culture is about 26 ºC (Joshi *et al.,* 2013). The pH of the medium is also an important factor for tissue culture. The pH of the medium is usually adjusted to between 5 and 5.8 before autoclaving and extremes of pH are avoided. Light and temperature will give effect in humidity of the culture vessel and pH of the medium plays a role in osmotic potential of the medium. Mantaining humidity and osmotic potential is very important due to its capability to affect the growth and development of plantlets *in vitro* in different ways (Kataria *et al.,* 2013).

2.13 Sugarcane micropropagation

Sugarcane is a perennial grass that normally reproduces vegetatively through nodal buds and rhizomes but seed propagation also occurs. Commercial sugarcane is propagated vegetatively by nodal cuttings and for this reason, micropropagation offers a practical and fast method for mass production of clonal material (Bakker, 1999). *In vitro* techniques for the mass propagation of healthy sugarcane plantlets can be achived via organogenic and/or somatic embryogenic (direct and indirect) pathways (Synman *et al.*, 2010).

2.13.1 Callus formation in sugarcane micropropagation

Callus can be initiated from any sugarcane tissue such as root apical meristems, young root, leaves, node tissue, immature florescence, pith and parenchyma (Patil *et al*., 2010). But present investigation demonstrates that inner fresh leaves and shoot apical meristem of sugarcane are highly amenable to *in vitro* callus culture (Ali *et al*., 2008). According to Tiwari (2013) callus volume is found to be larger for the young leaf rather than the apical meristem explants.

Among the auxins presented, 2,4-D at 3.0 mg/l is more potent for callus induction than other auxin hormones (Ali *et al.*, 2008). Yellow callus is typically

produced from 2,4-D containing culture media. Beside its amenability, the *in vitro* sugarcane regenerated from callus is susceptible to somaclonal variation for different traits like high yield, more sugar recovery, disesase resistance, early maturity and drough tolerant (Ali *et al*., 2012).

2.13.2 Shoot regeneration in sugarcane *in vitro*

Shoot regeneration of sugarcane can be achived by both organogenesis and somatic embryogenesis (Khan and Khatri, 2006). In most cases, shoot regeneration of sugarcane are come from callus culture also known as organogenesis (Yadav and Ahmad, 2013). According to Tarique *et al.* (2010), shoot regeneration from sugarcane callus was first demonstrated by Heinz and Mee in 1969. High level of cytokinin and low level of auxin is essential for regeneration of shoots in sugarcane leaf sheath callus (Smiullah *et al*., 2013). Combination between BAP, kinetin and NAA mostly give the best response in shoot regeneration of sugarcane (Yadav and Ahmad, 2013). Callus can also be transferred to 9.3 mM kinetin and 22.3 mM α-naphthaleneacetic acid (NAA) to obtain rapid regeneration of shoot (Chengalrayan and Gallo-Meagher, 2001). However, thidiazuron aslo known as TDZ recently becomes superior plant growth regulator to other cytokinins tested for shoot regeneration of sugarcane from callus. TDZ treatments give faster shoot regeneration than the kinetin/NAA treatment (Gallo-Meagher *et al*., 2000).

III. MATERIALS AND METHODS

3.1 Date and place

The special problem reseach entitled “Callus Induction and Tillering Capability of 4 Sugarcane Cultivars (*Saccharum officinarum* L.) under *In Vitro* Culture” was conducted on November 12th 2013 at Tissue Culture Laboratory, Center for Agricultural Biotechnology, Kasetsart University Kamphaeng Saen Campus.

3.2 Materials

Healthy leaves (innermost spindle leaf) of 4 field-grown sugarcane cultivars, K92-80, KK3, LK 95-127 and K 93-219 were used as special problem material. MS (Murashige and Skoog) medium was used with additional of 2,4-D and coconut water as plant growth regulator. Sucrose was given as carbohydrate source. All of the ingradients were solidified using agar powder.

3.3 Methods

3.3.1 Explant collection

The cane top containing young leaves of 4 field-grown sugarcane cultivars, K92-80, KK3, LK95-127 and K93-219 were cut approximately 20–30 cm below the uppermost internode of sugarcane.

3.3.2 Surface sterilization

The outer whorls of cane tops were removed and remaining 1-2 centimeters in diamater of immature leaf segments. The explants were surface sterilized with 20% and 15% of comercial bleach for 10 minutes each and subsequently rinsed with steriled water 3 times for 5 minutes each.

3.3.3 Callus induction

Surface sterilized immature leaf segments were used for callus induction. The outer two or three whorls of leaves were aseptically cut and removed remaining innermost whorls containing more or less 2 mm in diameter of immature leaf. Immature leaves segments were cut into 0.5 cm-long in aseptic

condition. Each cultivar has 10 replications which was used in callus induction stage. The callus induction medium was MS (Murashige and Skoog) medium supplemented with 3.0 mg/L of 2,4-D, 2% of sucrose, 10% (V/V) of coconut water and 0.7 % of agar. The pH of the medium was adjusted to 5.7 and autoclaved at 121^{o} C for 15 minutes. Callus induction was initiated under complete darkness at 25^{o} C ± 1 for 60 days. The calli were subcultured to the fresh medium every 30 days.

3.3.4 Shoot regeneration

The healthy and uncontamined calli were transferred onto plant regeneration medium. The calli were first cut into 0.5–1 cm each. There were 11 replications in each cultivar used in this stage. Shoot regeneration medium was MS (Murashige and Skoog) containing 10% (V/V) of coconut water for plant growth regulator. The MS medium also suplemented with 2% of sucrose as carbon source and 0.7% agar as solidifying agent. Explants were cultured under white florescent light with intensity of 55 $\mu M.m^{-2}.s^{-1}$ and 16 hours photoperiod at 25°C ± 1. The explants were subcultured to the fresh medium every 30 days.

3.3.5 Tillering capability

The healthy and uncontamined shoots that had 2-4 cm in height were separated into a single shoot. Each shoot was transferred to tillering induction medium. There were 11 replications in each cultivar used in this stage. The medium used was MS (Murashige and Skoog) containing 10% of coconut water for plant growth regulator. The MS medium also suplemented with 2% of sucrose as carbon source and 0.7% agar as solidifying agent. Explants were cultured under white florescent light with intensity of 55 $\mu M.m^{-2}.s^{-1}$ and 16 hours photoperiod at 25°C ± 1. The explants were subcultured to the fresh medium every 30 days.

3.3.6 Data collection and statistical analysis

The data collection were callus physical properties and callus induction percentage. They were done every 3 weeks for 2 months from November 12th 2013 to January 10th 2014.

The data collected in shoot regeneration medium were shoot physical properties, number of callus producing shoot (≥ 2 cm) and average number of shoots produced (≥ 1 cm). All the data were collected every 1 week for 2 months from January 11th to March 15th 2014.

In tillering capability stage, data collected were tiller physical properties, number explant producing tiller (≥ 1cm) and average number of tillers produced (≥ 1 cm). They were recorded every 2 weeks for 2 months from March 15th to Mei 12th 2014.

A completely randomized design (CRD) was used with 4 different sugarcane cultivars. The data of callus induction, shoot regeneration and tillering capability were collected and analyzed using ANOVA statistical analysis to find out the significant effects of the source variables. Duncan's multiple range test (DMRT) was futher applied to the data to test the significant differences between the treatment means ($p \leq 0.05$).