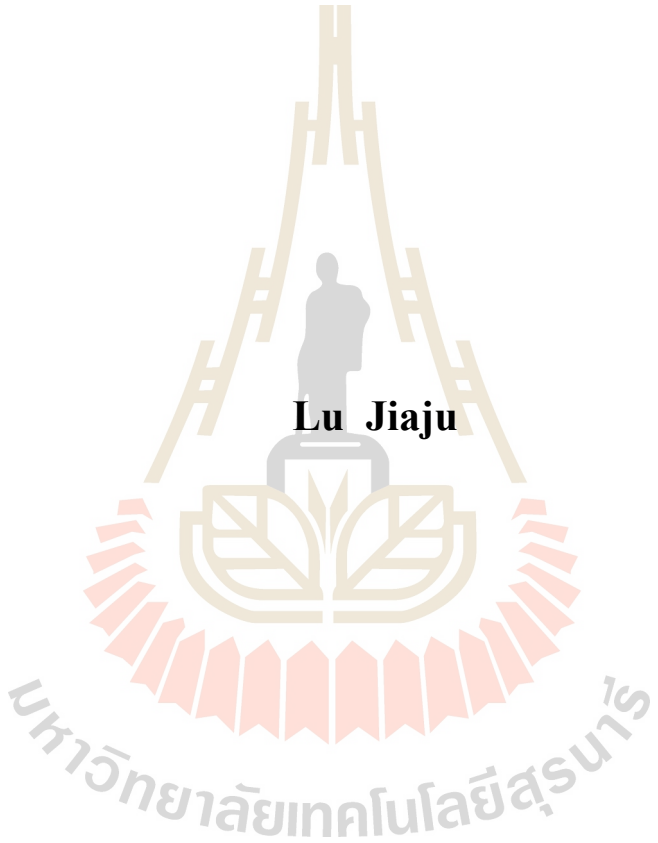
DEVELOPMENT OF LOW-COST OPEN TISSUE CULTURE TECHNOLOGY FOR SUGARCANE

Lu Jiaju



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การพัฒนาเทคโนโลยีเพาะเลี้ยงเนื้อเยื่ออ้อยแบบระบบเปิดต้นทุนต่ำ

นายหลู จาจุ่ย

วิทยานิพนธ์นี้สำหรับการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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DEVELOPMENT OF LOW-COST OPEN TISSUE CULTURE TECHNOLOGY FOR SUGARCANE

Suranaree University of Technology has approved this thesis submitted in fulfillment of the requirements for the Degree of Doctor of Philosophy.

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นายหลู จาจุ่ย : การพัฒนาเทคโนโลยีเพาะเลี้ยงเนื้อเยื่ออ้อยแบบระบบเปิดต้นทุนต่ำ (DEVELOPMENT OF LOW-COST OPEN TISSUE CULTURE TECHNOLOGY FOR SUGARCANE) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.อารักษ์ ธีรอำพน, 123 หน้า.

วัตถุประสงค์ของงานวิจัยนี้คือ เพื่อพัฒนาเทคโนโลยีเพาะเลี้ยงเนื้อเยื่อแบบระบบเปิดต้นทุนต่ำสำหรับการขยายพันธุ์อ้อยโดยผสมผสานการใช้สารควบคุมจุลินทรีย์ประเภทออกฤทธิ์กว้างร่วมกับอาหารเพาะเลี้ยงเนื้อเยื่อปราศจากน้ำตาลและปุ๋ยแก๊สคาร์บอนไดออกไซด์ การทดลองเริ่มจากการคัดกรองหาเชื้อจุลินทรีย์ที่มักพบปนเปื้อนอาหารเพาะเลี้ยงเนื้อเยื่อสูตร MS ในระหว่างการเพาะเลี้ยงเนื้อเยื่ออ้อย พบจุลินทรีย์จำนวน 6 ชนิด ได้แก่ *Mucor* sp, *Rhizopus* sp, *Aspergillus flavus, Alternalia* sp, *Penicillium* sp และ *Bacillus* sp. จากนั้นทำการทดลองหาชนิดของสารสกัดอินทรีย์จากพืชและสารเคมีที่ระดับความเข้มข้นต่าง ๆ เพื่อควบคุมจุลินทรีย์แต่ละชนิดที่คัดกรองได้ พบว่า นิโคติน 40 มิลลิกรัมต่อลิตร สารสกัดจากกระเทียม 80 ไมโครกรัมต่อลิตร (57.1%) คาร์เบนดาซิม 48 มิลลิกรัมต่อลิตร และ NaOCl 14.5 มิลลิกรัมต่อลิตร (Yi Peilong) สามารถควบคุมการปนเปื้อนเชื้อจุลินทรีย์ในอาหาร MS ได้ดีที่สุดแต่มีความเป็นพิษต่อเนื้อเยื่ออ้อยน้อยที่สุดและตั้งชื่อ Qiaxing No.1 ให้กับส่วนผสมสูตรดังกล่าว การทดลองเพาะเลี้ยงเนื้อเยื่ออ้อยในสูตรอาหาร MS ที่ไม่ได้นึ่งฆ่าเชื้อ โดยมีการเติม 6-BA 2.5 มิลลิกรัมต่อลิตร และ Qiaxing No.1 ความเข้มข้น 0.5% (ปริมาตรต่อปริมาตร) จากสต็อกความเข้มข้น 200 เท่า พบว่า สามารถควบคุมการปนเปื้อนจุลินทรีย์ให้เหลือเพียง 6.67% โดยมีการรอดชีวิตของเนื้อเยื่ออ้อยถึง 80% เมื่อเทียบกับการเพาะเลี้ยงเนื้อเยื่ออ้อยแบบปกติ ที่มีการปนเปื้อน 43.33% และการรอดชีวิตของเนื้อเยื่ออ้อย 56.67%. ในช่วงระยะการเพิ่ม จำนวนต้นกล้าของกระบวนการย้ายเนื้อเยื่ออ้อยแบบระบบเปิด ในสูตรอาหาร MS ปราศจากน้ำตาลที่เติม 6-BA 1 มิลลิกรัมต่อลิตร ไคเนติน 0.5 มิลลิกรัมต่อลิตร และ 200X Qiaxing No.1 0.5% (ปริมาตรต่อปริมาตร) พร้อมทั้งเพิ่มปุ๋ยแก๊สคาร์บอนไดออกไซด์ 0.1 กรัม (เทียบเท่าแก๊สคาร์บอนไดออกไซด์ 2,144 ไมโครลิตรต่อลิตร ในขวดเพาะเลี้ยงเนื้อเยื่อขนาด 250 มิลลิลิตร) โดยออกแบบพิเศษไม่ให้เม็ดปุ๋ยสัมผัสกับอาหารเพาะเลี้ยงเนื้อเยื่อ พบว่า สามารถเพิ่มประสิทธิภาพการแตกหน่อได้ในระดับ 3.15 และลดการปนเปื้อนของจุลินทรีย์เหลือเพียง 3.5% เมื่อเทียบกับการเพาะเลี้ยงเนื้อเยื่อแบบปกติที่มีประสิทธิภาพ 3.12 และมีการปนเปื้อนของจุลินทรีย์ 7% หลังการย้ายเนื้อเยื่ออ้อย 20 วัน ส่วนในช่วงระยะการชักนำให้เกิดรากที่ใช้สภาพการทดลองเดียวกันกับในช่วงระยะการเพิ่มจำนวนต้นกล้า แต่ปรับเปลี่ยนจากฮอร์โมน 6-BA และไคเนติน เป็น NAA 3 มิลลิกรัมต่อลิตร และปรับลดวุ้นเหลือ 4 กรัมต่อลิตร พบว่าเนื้อเยื่ออ้อยในสภาพการเพาะเลี้ยงดังกล่าว มีค่าอัตราการสังเคราะห์แสงสุทธิและน้ำหนักต้นสูงกว่าระบบการเพาะเลี้ยงเนื้อเยื่อแบบปกติ อีกทั้งมีค่าอัตรา

การอยู่รอดของต้นกล้าอ้อยสูงถึง 96.7% เมื่อเทียบกับระบบการเพาะเลี้ยงเนื้อเยื่ออ้อยปกติที่มีค่า 92.9% โดยมีการปนเปื้อนของจุลินทรีย์เพียง 3.5% ขณะที่ระบบการเพาะเลี้ยงเนื้อเยื่ออ้อยแบบปกติมีการปนเปื้อน 7.1% จากการคำนวณต้นทุนการผลิตต้นกล้าอ้อยจำนวน 10,000 ต้น พบว่า ระบบการเพาะเลี้ยงเนื้อเยื่อแบบเปิดสามารถผลิตกล้าอ้อยได้ในราคาเพียง 0.6 หยวนต่อต้น เมื่อเทียบกับการเพาะเลี้ยงเนื้อเยื่อแบบปกติที่มีต้นทุนถึง 1 หยวนต่อต้น ทำให้สามารถลดค่าใช้จ่ายได้ถึง 40%

สาขาวิชาเทคโนโลยีการผลิตพืช ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2557 ลายมือชื่ออาจารย์ที่ปรึกษา_____

LU JIAJU : DEVELOPMENT OF LOW-COST OPEN TISSUE CULTURE TECHNOLOGY FOR SUGARCANE. THESIS ADVISOR : ASST. PROF. ARAK TIRA-UMPHON, Ph.D., 123 PP.

SUGARCANE/OPEN TISSUE CULTURE/PHOTO-AUTOTROPHY/ ANTIMICROBIAL COMPOUNDS/CO_2 GAS FERTILIZER/LOW-COST

The main objective of this research was to develop a low-cost open tissue culture technology for sugarcane micropropagation using a combination of broad-spectrum antimicrobial compounds, sugar-free medium and CO_2 gas fertilizer. After extensive screening of sugarcane "*Qiantang* No.5" on a basal MS medium, six common microbes were found causing contamination in the tissue culture media, i.e. *Mucor* sp, *Alternaria* sp, *Penicillium* sp, *Aspergillus flavus*, *Rhizopus* sp and *Bacillus* sp. Formulations of various antimicrobial compounds to inhibit these microbes were screened for open tissue culturing of sugarcane. Qianxing No.1 comprising of 40 mg/L nicotine, 80 µl/L garlic extract (57.1% w/v conc), 48 mg/L active carbendazim and 14.5 mg/L NaOCl (Yi Peilong) was selected because of its best inhibiting microbial contamination and less toxic to the explants. The non-autoclaved MS medium was mixed with 5 ml/L 200X Qianxing No.1 (0.5% v/v) and 2.5 mg/L 6-BA gave only 6.67% microbial contamination with 80% survival rate of the explants after 15 days of induction phase compared to 43.33% contamination and 56.67% survival rate obtained from the autoclaved MS medium. The tissue transplanting process using the medium supplemented with Qianxing No.1 was carried out under an open condition outside an aseptic chamber. The technique was subsequently developed by supplying CO_2 gas fertilizer into tissue

culture vessels to increase plantlet photosynthetic capacity and to allow the use of sugar-free medium to lower the chance of microbial contamination during open tissue culture. On day 20 of the multiplication phase, the non-autoclaved MS medium supplemented with 1 mg/L 6-BA, 0.5 mg/L Kinetin and 5ml/L 200X Qianxing No.1, enriched with 1 g CO_2 gas fertilizer (2,144 ul/L CO_2 gas) in 250 ml specially designed culture bottles could improve propagation efficiency from 3.12 to 3.15, and decrease the contamination from 7% to 3.5% comparing with the conventional operation. On day 25 of the rooting phase with similar setting using the non-autoclaved MS medium supplemented with 3 mg/L NAA and 4 g/L agar, the net photosynthetic capacity and plantlet (fresh) weight were much higher than those of the conventional method. This developed open tissue culture method also showed better plantlet survival rate of 96.7%, with 3.5% contamination compared with 92.9% and 7.1%, respectively, obtained from the conventional method. The cost of plantlet production using the developed open tissue culture technology was also decreased to RMB￥0.6 per plantlet compared with that of RMB￥1.0 per plantlet for the production of 10,000 sugarcane plantlets.

School of Crop Production Technology Student's Signature_____

Academic Year 2014 Advisor's Signature_____

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LIST OF ABBREVATION

SCMV	=	*Sugarcane Mosaic Virus*
SrMV	=	*Sorghum Mosaic Virus*
SCSMV	=	*Sugarcane Streak Mosaic Virus*
RSD	=	*Ratoon Stunting Disease*
PPF	=	photosynthetic photon flux
v/v	=	volume/volume
(μ, m)L,g	=	(micro, milli)Liter, gram
μg	=	micro gram
μm	=	micro meter
WP	=	wettable powder
ppm	=	part per million
μmol	=	micro mole
w/v	=	wight/volume
NCM	=	nutrient-circulated photoautotrophic micropropagation
WUE	=	water use efficiency
MS	=	Murashige and Skoog medium
CRD	=	Complete Randomized Design
TRT	=	treatment
atm	=	atmosphere
UV	=	ultraviolet

LIST OF ABBREVATION (Continued)

SPSS	=	Statistic Package for Social Science
CV	=	coefficient of variation
KT	=	kinetin
NAA	=	1-naphthylacetic acid
6-AB	=	6-benzilaminopurine
DNA	=	Deoxyribose Nucleic Acid
IRGA	=	Infrared gas analyzer
Pn	=	Net photosynthetic rate

CHAPTER I

INTRODUCTION

1.1 Introduction

Sugarcane (*Saccharum* spp) belonging to the grass family, is one of the most important economic crops in China. Its planting acreage reached 1.586 million hectares in 2012, accounting for 92% of the total sugar crops. Furthermore, sugarcane can provide several valuable products, such as sugar, ethanol and electricity (Luo *et al.,* 2014). At present, two serious sugarcane diseases have been observed, including *Sugarcane Mosaic Virus* (SCMV) (Wang *et al.,* 2009) and *Ratoon Stunting Disease* (RSD) (Li *et al.,* 2014; Xu *et al.,* 2008). To be specific, when the incidence of SCMV reaches 30%, then the yield loss can be 3-50%. This case can be seen particularly in Guangxi province, China (Wang *et al.,* 2004; XuPark *et al.,* 2008). As for the RSD, if the average infection rate reaches 50%, then the yield loss will be about 12-37%. Taken drought and RSD infection together consideration, the loss can be high as 60%, perhaps causing the sugar content to decline to 0.5% (absolute value) under the drought condition, particularly, in Guangxi and Guizhou provinces in China (Comstock, 2002; Deng *et al.,* 2004; Lu *et al.,* 2007; Shen *et al.,* 2007; Shen *et al.,* 2006; Wang, 2007). For these reasons, it has become urgent to improve the sugarcane yield in the limited acreage of China. rapid development of sugarcane industry by

growing disease resistant varieties and Two methods have been employed to ensure the disease-free planting stock obtained from plant tissue culture technology. Among the two, the first method could be the best choice but the second is also a good alternative.

Plant tissue culture technology is a popular way to produce plantlets in a short time. It allows rapid reproduction of disease-free plants of genetically identical origin. Single cells, protoplasts, pieces of leaves, shoots, or less commonly roots can be used to generate a new plant on culture media and be given the required nutrients and plant hormones (George and Manuel, 2013). Sugarcane micropropagation has been routinely used in order to produce healthy plantlets from new improved cultivars, which can be more readily available to farmers. However, with the widely use of plant tissue culture technology, some problems are arising. First, the excess of sucrose concentration in the media not only might cause cellular dehydration by osmotic gradient, but also lead to higher a microbial contamination (Perez *et al.,* 2004). And second, for maintaining disinfection and sterilization of the culture media, autoclaving is generally performed, consuming large quantity of electricity (Ahloowalia and Savangikar, 2004; George and Manuel, 2013).

In order to overcome these problems, numerous reports have been published on the reduced microbial contamination rate and enhanced growth of plantlets *in vitro* by using sugar-free media, increasing photosynthetic photon flux (PPF) and the CO_2 concentration in the vessel on carnation (Kozai and Iwanami, 1988), on Eucalyptus (Kirdmanee *et al.,* 1995), on potato (Niu and Kozai, 1997) and on statice (Xiao and Kozai, 2006a; Xiao *et al.,* 2011). Furthermore, reports on plant open tissue culture technique have also been published on the reduced cost per plantlet by using

antimicrobial compounds (chemical, botanical, or combination of both) instead of autoclaving to sterilize the media and prevent microbial contamination, hence saving the electricity expenses and allowing the operation to be done in an open condition. These reports include the studies carried out by Chen (2004) and Cui (2005) on grape, apple, potato and butterfly orchid. Sawant and Tawar used sodium hypochlorite as media sterilant in sugarcane micropropagation (Sawant and Tawar, 2011) and Lu (2012) used antimicrobial compounds Qianxing No.1 in sugarcane open tissue culture (Lu *et al.,* 2012).

In spite of the advantages mentioned above, there are still some defects of sugar-free and open tissue cultures. For the sugar-free tissue culture, although autoclaved sugar-free media are contributing to lower contamination, the operation steps have to be conducted on clean benches and the complicated CO_2 gas applies system is expensive (Guan, 2007). In terms of open tissue culture, antimicrobial compounds were supplemented in non-autoclaved media, although the operation steps could layout in a relative aseptic lab rather than on clean benches, but the sugar contained in the media could still support the microbial growth. These are the reasons why open and sugar-free tissue culture technologies have not been, so far, widely used until now.

Plant species are autotrophic. Previous studies displayed an increase in sugarcane photosynthesis grown under elevated CO_2 (De Souza *et al.,* 2008; Vu and Allen Jr, 2009a; Vu and Allen Jr, 2009b; Vu *et al.,* 2006a). But most of them could not express such property when cultivated *in vitro*, because of the low CO_2 supply and low gas exchange inside the culture flask (Kozai *et al.,* 2005). CO_2 gas fertilizer, an industrial product, releases CO_2 gas slowly when stimulated by artificial light or sun

light, and is suitable for plant photosynthesis (Min *et al.,* 2001; Wen *et al.,* 2002). However, there is no reports of using CO_2 gas fertilizer for sugarcane micropr-opagation, although the application of CO_2 gas fertilizer in greenhouse has been quite successful in producing vegetables (Li, 2007a; Li and Zhou, 2004; Li, 2009).

The objective of this study was to combine the advantages of sugar-free tissue culture with that of the open tissue culture, as well as to overcome their defects by the application of CO_2 gas fertilizer and a broad-spectrum antimicrobial compounds in the open sugar-free tissue culture.

1.2 Significance of the Study

The low-cost open tissue culture technology takes benefits from the broad-spectrum antimicrobial compounds and CO_2 gas fertilizer as follows: First, the antimicrobial compounds contained in media does not need autoclaving and aseptic transferring chamber to prevent microbial contamination, hence lower the cost of operation. Second, the process of plant tissue culture would become very simple and would be conducted in a common culture room, which can save labor cost and enhance working efficiency. Third, the survival rate and quality of plantlets would be improved, which can reduce the unit price of plantlets.

In the future, indirect benefits of the technology would be included as follows: First, in education field, the open sugar-free tissue culture technology can be utilized to help students to do plant clone experiment in classroom or their home. Second, in the propagation field of high value economic plant, the technology could improve the quantity and quality of plantlets, hence enhancing its competitive power in the international markets. Third, in transgenic research field, the broad-spectrum Qianxing

No.1 could simplify the process of transgenic, therefore saving time and improving the efficiency of experiment.

1.3 Research Objectives

1. To select and test broad-spectrum antimicrobial compounds for inhibition of microbial contamination in sugarcane tissue culture media.

2. To determine the effect of antimicrobial compounds on the growth and development of sugarcane explants in open tissue culture.

3. To determine the combination of antimicrobial compounds and CO_2 gas fertilizer on the growth and development of sugarcane explants in sugar-free media.

4. To evaluate efficiency of the low-cost tissue culture technology in sugarcane micropropagation.

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CHAPTER II

REVIEW OF LITERATURE

2.1 General Information of Sugarcane

Sugarcane (*Saccharum* spp) is a tropical, perennial grass that forms lateral shoots at the base to produce multiple stems, typically three to four meters high and about five centimeter in diameter. The stems grow into cane stalk, which constitutes approximately 75% of the entire plant when mature. A mature stalk is typically composed of 11-16% fiber, 12-16% soluble sugars, 2-3% non-sugars, and 63-73% water (Kim and Dale, 2004). The sugarcane crop is sensitive to the climate, soil type, irrigation, fertilizers, insects, disease control, varieties, and the harvest period. The average yield of cane stalk is 60-70 tons per hectare per year. However, this yield can vary between 30 and 180 tons per hectare depending on varieties and crop management approaches used in sugarcane cultivation (Blackburn, 1984; James, 2004; Wikipedia, 2014).

Sugarcane is the world's largest crop by production quantity. In 2012, The Food and Agriculture Organization of the United Nations estimated that it is cultivated on about 26.0 million hectares, in more than 90 countries, with a worldwide harvest of 1.83 billion tons. Brazil was the largest producer of sugarcane in the world. The next five major producers, in decreasing amounts of production, are India, China, Thailand, Pakistan and Mexico (Smart, 2013).

Sugarcane is one of the most important economic crops in China, providing several valuable products and sub-products, such as sugar, ethanol and electricity (Kim and Dale, 2004). Its planting acreage accounted for 92% of the total sugar crops and reached 1.586 million hectares in 2012 (Luo *et al.,* 2014). *Sugarcane Mosaic Virus* (SCMV) (Wang *et al.,* 2009) and *Ratoon Stunting Disease* (RSD) (Li *et al.,* 2014; Xu *et al.,* 2008) are two serious sugarcane diseases all over the world. Sugarcane ratoon stunting disease (RSD) caused by *Leifsonia xyli* subsp. *xyli* (Lxx), is one of the most serious diseases that limits sugarcane production worldwide. Li *et al* (2014) reported that under the field condition, the main cultivars grown over large areas, including Guitang 94-119, Yuetang 93-159, Yuetang 00-236 and Guitang 11 showed high RSD incidence rates in China, suggesting that the focus on these cultivars and that they should be replaces by the healthy, bacteria-free seedlings (Li *et al.,* 2014). Sugarcane mosaic disease has become one of the most serious and prevalent diseases in China. In the cane-growing regions of southern China, especially in Yunnan and Guangxi provinces, disease incidence is generally over 30% and can be as high as 100% in susceptible cultivars in the worst infected fields, causing a decrease of 11-35% in seed cane germination rate, culminating in a loss of 3-50% of the total cane yield and a decrease of 6-14% in the sucrose content, and equating to monetary losses of over US$ 30 million annually (Huang and Li, 2011; Huang *et al.,* 2007). Under natural conditions, sugarcane mosaic disease can be caused by *Sugarcane mosaic virus* (SCMV), *Sorghum mosaic virus* (SrMV) and/or *Sugarcane streak mosaic virus* (SCSMV), all of which belong to the Potyviridae family **(**Chatenet *et al.,* 2005; Chen *et al.,* 2002; Seifers *et al.,* 2000).

2.2 Conventional Plant Tissue Culture

Cell totipotency theory was reported by Haberlandt on 1902. Cell totipotency is the ability of a single cell to divide and produce all of the differentiated cells in an organism, including extraembryonic tissues (Mitalipov and Wolf, 2009). Plant tissue culture, or the aseptic culture of cells, tissues, organs, and their components under defined physical and chemical conditions *in vitro*, is an important tool in both basic and applied studies as well as in commercial application. It owes its origin to the ideas of theoretical basis for plant tissue culture proposed by Gottlieb Haberlandt in his address to the German Academy of Science on his experiments on the culture of single cells (Thorpe, 2007). Plant tissue culture is used widely in plant research domain; it also has a number of commercial applications included as follows:

(1) Micropropagation is widely used in forestry and in floriculture (Ezekiel, 2010). Micropropagation can also be used to conserve rare or endangered plant species (Pence, 1999).

(2) A plant breeder may use tissue culture to screen cells rather than plants for advantageous characters, herbicide resistance/tolerance e.g. (Van den Bulk, 1991).

(3) Large-scale growth of plant cells in liquid culture in bioreactors is for production of valuable compounds, like plant-derived secondary metabolites and recombinant proteins used as biopharmaceuticals (Georgiev *et al.*, 2009).

(4) Micropropagation is applied to sugarcane related species cross by protoplast fusion and regeneration of the novel hybrid (Liu and Chen, 1976; Wieczorek and Wright, 2012).

(5) It is used for production of doubled monoploid (dihaploid) plants from haploid cultures by anther culture or by pollen culture to achieve homozygous lines

more rapidly in breeding programmers, usually by treatment with colchicine which causes doubling of the chromosome number (Asif, 2013).

(6) As a tissue for transformation, it is followed by either short-term testing of genetic constructs or regeneration of transgenic plants (Purkayastha *et al.*, 2010).

(7) Certain techniques such as meristem tip culture can be used to produce virus-free plant material from viruses stock, such as potatoes and many species of soft fruit. Virus-free potatoes could increase yields 42-62% (Zhen *et al.*, 2010).

(8) Micropropagation uses meristem and shoots culture to produce large numbers of identical individuals. Since 1980, in order to reproduce ornamental plants with higher economic value, many countries have built their plant tissue culture companies. These companies have the ability to reproduce more than100 thousand seedlings *in vitro* per year. The Western Europe reached an annual output of more than 200 million tissue culture plants (Fan and Zhan, 1996).

However, with the wide use of plant tissue culture technology, some defects of conventional plant tissue culture technology are arising as following: the conventional plant tissue culture technique was mostly carried out using small culture vessels with agar or other gelling agents containing nutrients and sucrose as a carbon source for the plantlets at a low photosynthetic photon flux (PPF). The environment of conventional plant tissue culture system *in vitro* is characterized by high relative humidity, high ethylene concentration, stagnant air, and a low CO_2 concentration in the vessel during the photoperiod (Fujiwara and Kozai, 1995). This environment *in vitro*, which is entirely different from the ex vitro environment such as the greenhouse environment, often causes malfunction of stomata, poor epicuticular wax development, elongated shoots, low chlorophyll concentration, hyperhydration of plantlets, low growth rate,

little rooting, callus formation at the base of explants and low percent survival ex vitro (Majada *et al.*, 2002; Serret *et al.*, 1996). Moreover, excess of sucrose concentration in the media not only might cause cellular dehydration by osmotic gradient, but also lead to higher microbes contaminations (Perez *et al.*, 2004). Furthermore, for maintaining disinfection and sterilization of the culture media, autoclaving is generally performed, consuming large quantity of electricity (Ahloowalia and Savangikar, 2004; George and Manuel, 2013).

2.3 Plant Sugar-free Tissue Culture

2.3.1 Concept of plant sugar-free tissue culture

In order to solve those problems as described above, plant sugar-free tissue culture was developed by Kozai *et al* (Kozai, 1991; Kozai *et al.*, 1988). Plant sugar-free tissue culture is narrowly defined as the micropropagation without sugar in the culture media, in which the growth or accumulation of carbohydrates of plantlets are dependent fully upon photosynthesis and inorganic nutrient uptake (Kozai, 1991; Kozai and Kubota, 2001; Zobayed *et al.*, 2004). Thus, it can also be called photosynthetic micropropagation, inorganic micropropagation, or micropropagation in sugar-free medium **(**Kozai *et al.*, 2005). Maintaining the environmental conditions *in vitro* (e.g., levels of PPF, CO_2 concentrations, etc.) at optimal ranges is critical.

2.3.2 Application of plant sugar-free tissue culture

A number of reports have been published on the reduced microbial contamination rate and enhanced growth of plantlets *in vitro* by using sugar-free media, increasing photosynthetic photon flux (PPF) and the CO_2 concentration in the vessel. First of all, Kozai *et al* (1988) successfully cultured potato (*Solanum*

tuberosum L.) plantlets in sugar-free media with a goal to develop an automated mass propagation system for producing disease-free seed potato tubers and disease-free potato plantlets (Kozai *et al.*, 1988). Other reports, including the studies by Kozai and Iwanami (1988), their research results indicated that carnation (*Dianthus caryophyllus L.*) explants were cultured in acclimatization stage with CO_2 concentration at 1000-1500 ppm and a photosynthetic photon flux of 150 μmol $m^{-2}s^{-1}$ (400-700 nm). The plantlet growth was promoted to a large extent by CO_2 enrichment under high photon flux. The order of largest to smallest of fresh and dry weight increases of the plantlets during the culture was CO_2 enriched treatment with 1.0 % sucrose, CO_2 enriched treatment with 0.0 % sucrose, CO_2 enriched treatment with 2.0 % sucrose (Kozai and Iwanami, 1988). Wu and Lin (2013) reported that the improvements in survival percentage and *ex vitro* growth of *Protea cynaroides* L. plantlets were the result of their enhanced photosynthetic ability *in vitro*, which resulted in the production of high-quality plantlets. Significant improvements in the overall growth of *Protea cynaroides* L plantlets were achieved through the use of photoautotrophic micropropagation with 10000 μmol·mol^{-1} CO_2 enrichment (Wu and Lin, 2013). Saldanha *et al* (2013) found that CO_2 enrichment (360 or 720 μmol mol^{-1}) led to increased photosynthetic pigments and reduced stomatal density of *in vitro* cultivated *P. glomerata* **(**Saldanha *et al.*, 2013). Oh *et al* (2013) reported that combinations of vermiculite and perlite with different particle sizes made different physical and chemical properties, and some of these mixtures improved growth of potato plantlets in the nutrient-circulated photoautotrophic micropropagation (NCM) system **(**Oh *et al.*, 2012). Xiao *et al* (2003) successfully cultured sugarcane explants in sugar-free MS liquid media with double-strength KH_2PO_4, $MgSO_4$, $FeSO_4$, and Na_2-EDTA in

the vessel with enhanced natural ventilation when CO_2 concentration in the culture room was kept at 1500 μmol mol^{-1}(four times the atmospheric CO_2 concentration) during the photoperiod (Xiao *et al.*, 2003). DaSilva *et al* (2006) also reported that the best plantlets of *Spathiphyllum* cv. Merry was obtained by culturing the plantlets in sugar-free liquid media using Vitron vessel under a CO_2 concentration of 3,000 μmol mol^{-1} and a PPF of 45 μmol $m^{-2}s^{-1}$ (Da Silva *et al.*, 2006).

2.3.3 Advantages and disadvantages of plant sugar-free tissue culture

As described by Kozai and Kubota (2005), photoautotrophic micropropagation has many advantages over conventional micropropagation with respect to improvement of plantlet physiology (biological aspect) and the operation or management in the production process (engineering aspect). The advantages of biological aspects include:

(1) Promotion of growth and photosynthesis;

(2) High survival percentage/smooth transition to ex vitro environment;

(3) Elimination of morphological and physiological disorders;

(4) No callus formation at the base of explants;

(5) Little loss of plantlets due to microbial contamination.

Advantages of engineering aspects include :

(1) Flexibility in the design of the vessel (larger vessels);

(2) Increased annual productivity per floor area;

(3) Reduction in labor cost;

(4) Simplification of the micropropagation system.

The following items are often considered as disadvantages of photoautotrophic micropropagation :

(1) Relative complexity of techniques and knowledge required for controlling *in vitro* environment;

(2) Expense for lighting, CO_2 enrichment, and cooling;

(3) Limitation of application to multiplication systems using multiple buds or shoots.

(4) The whole process has to be conducted in germfree condition, where the equipments supply CO_2 gas for tissue culture container is expensive (Guan, 2007).

2.3.4 Summary of plant sugar-free tissue culture

Numerous studies have been conducted on improving the *in vitro* environment and enhancing the growth and development of cultures/plantlets since the concept of plant sugar-free tissue culture (photoautotrophic micropropagation) was developed more than two decades ago. For successful photoautotrophic micropropagation, it is critical to control *in vitro* environment for promoting photosynthesis, transpiration and nutrient uptake of plantlets. The carbohydrate for growth has to be produced by the culture itself in a photoautotrophic micropropagation system. Insufficient supply of CO_2 during photoperiod inhibits plantlet photosynthesis as supported by recent studies on myrtle plantlets (Lucchesini *et al.,* 2001; Lucchesini *et al.,* 2006). Therefore, the primary goal of environmental control in photoautotrophic micropropagation is to promote photosynthesis of plantlets. It is critical to maintain the *in vitro* environment such as CO_2 concentration and air current speed inside the vessel, temperature, and PPF at optimal ranges for maximum photosynthesis of the plantlets. The headspace of the ‘‘closed’’ culture vessel is the site in which temperature, light, and gases interact to determine the *in vitro* environment. Temperature and light control in a culture room is relatively easy. Therefore, the most challenging task is to increase the ventilation

rate of the culture vessel while keeping pathogen-free conditions to improve the *in vitro* environment and to minimize the differences in aerial environment between the *in vitro* and *ex vitro* (Xiao *et al.*, 2011).

2.4 Plant Open Tissue Culture

2.4.1 Concept of plant open tissue culture

Cui *et al* (2004) successfully developed a simple plant tissue culture system, which is narrowly defined as plant open tissue culture on the reduced cost per plantlet by using antimicrobials instead of autoclave sterilized media against microbial contamination, then saving electricity expenses and enhancing efficiency of inoculated plantlets in a relative aseptic inoculating lab rather than in clean bench and so on (Cui, 2005). The antimicrobials was made of several medicinal herbs by the theory of traditional Chinese medicine, it could inhibit most of bacteria and fungi in plant culture media, but just a slight harm on the growth and development of plantlets (Wei *et al.*, 2009; Zhang *et al.*, 2005). Thus, antimicrobials used during the whole process of open plant tissue culture could inhibit contamination under the condition of non-autoclaved media contioned antimicrobials, without autoclave and clean bench, plastic cup replaced glass culture bottle. The key point of open tissue culture is optimizing the concentration of antimicrobials in media to inhibit microbial contamination and controlling contamination rate lower than 10.0% (Cui, 2005; Cui *et al.*, 2004).

2.4.2 Microbial contamination and control by antibiotic substance in plant tissue culture

Bacteria and fungi contamination in plant tissue culture is a major problem in

plant tissue culture process. The most predominant fungus in plant tissue culture is follows *Aspergillus* sp, *Penicillium* sp, *Mucor* sp, *Rhizopus* sp, *Fusarium* sp, *Alternaria* sp, and *Candida* sp. The most predominantly occurring bacteria were *Bacillus* sp, *Micrococcus* sp, *Staphylococcus* sp, *Serratia* sp and *Pseudomonas* sp **(**Leifert *et al.*, 1994; Reed and Tanprasert, 1995).

To eliminate or avoid microbial contamination, it is essential to find the efficient sterilization methods. Firstly, laboratory must assess their situation, determine contamination source, and change their laboratory operations to avoid or eliminate most of the contaminants (Reed and Tanprasert, 1995). And then kill the microbe (epiphytic and entophytic) in plant by antibiotic substance. Epiphytic microbial may live in plant structures where disinfectants can not reach (Gunson and Spencer-Phillips, 1994). Entophytic microbe may be localized within the plant at cell junctions and the intercellular spaces of cortical parenchyma (Bunn and Tan, 2002). Conventional plant tissue culture is a highly sophisticated and expensive technology and poses some constraints which were mentioned above. Because of the cost per plant is generally very high, which depends on the production capacity of the laboratory, number of working staff, consumables and electricity expenses (Dookun *et al.*, 1996; Lal and Singh, 1997). Media sterilization through autoclaving requires a lot of power consumption, time and labor. Use of microwave oven, as an alternative of autoclave sterilization, has previously been attempted by some investigators (Keller *et al.*, 1988; Youssef and Amin, 2000), but the results were not satisfactory.

Normally, chemical disinfectants such as sodium hypochlorite, mercury bichloride, ethanol, hydrogen peroxide, bromine water, silver nitrate and antibiotics are generally used for surface sterilization of explants for raising shoot cultures *in vitro*

(Sawant and Tawar, 2011; Tiwari *et al.,* 2012). Kneifel and Leonhardt (1992) reported that with the two mixtures Imipenem/Ampicillin and Imipenem/Penicillin G at concentrations of 5 mg/L each, Gram-positive and Gram-negative (*Staphylococcus xylosus, S. aureus, S. cohnii, Bacillus* sp, *Corynebacterium* sp, *Pseudomonas esicularis*) bacterial growth inhibittion was most effective. It was proposed that these antibiotic mixtures can be applied advantageously to inhibit bacterial growth in tissue culture (Kneifel and Leonhardt, 1992). Bacterial and fungal contamination rates were 0% and the highest seedling survival rate was 60.0% when the concentration of carbendazim was 60.0 mg/L in Chinese rose tissue culture (Chen and Wu, 2014). In *Hevea brasiliensis* root culture, the contamination rate of explants decreased to 44.59 % when supplemented with 1.0 g/L carbendazim and 0.1% Yi Peilong in culture medium (Jiang *et al.,* 2011). Sawant and Tawar used sodium hypochlorite as media sterilant in sugar-cane tissue culture, they found that active chlorine concentration of 0.002% in the medium was effective in complete sterilization of the medium (Sawant and Tawar, 2011). But Cui noted that the application of chemical sterilant might lead to plantlets mutation, even though chemical sterilants might have better inhibition for microbes in plant culture media (Cui, 2005). Other researchers reported that botanical antimicrobials (such as garlic, nicotine, some activate ingredients of Chinese herbal medicine etc.) had better performance on resistance most of microbes, they were also more friendly to plantlet growth and development *in vitro* condition. For example, garlic has been used as a medicine since ancient times and has long been known to have antibacterial, antifungal and antiviral properties (Aala *et al.,* 2014; Bakri and Douglas, 2005; Harris *et al.,* 2001; Weiguo *et al.*, 2004). The garlic allicin with the concentration of 6.25 µg/ml and 12.5 µg/ml was highly efficient in inhibition of the growth of hyphal cells

(Aala *et al.*, 2014). Nicotine was very effective against bacterium, especially hose that cause disease, like the Staphylococcus. It can dissolve cell wall of micrococcus at the concentration of 1~5 %, in 120 minutes, the efficiency of nicotine to disinfect bacteria is as high as 89.9% (Hu, 2009). Growth of *Streptococcus mutants* cells was significantly repressed by 2.0-8.0 mg/ml of nicotine (Huang *et al.*, 2012).

Further more, Cui *et al* (2005) used Chinese traditional medicine theory, followed the Chinese herbal medicine combine principles of "the monarch and assistance relationship between Emperor and Minister in ancient China" extracted an antimicrobial substances (such as alkaloids, flavone and terpenoids, volatile oil, etc) from a variety of medicine plants. As a result, the steps of plant tissue culture could be simplified radically and the cost could be reduced greatly. There are some Chinese herbs as Table 2.1 shows (Bi *et al.*, 2009; Chen *et al.*, 2008; Xu *et al.*, 2008).

Table 2.1 Plant species and parts with antibacterial activities.

Plant name	**Active Part**	**Test microorganism**
Indigofera bungeana	Leaf	*Staphylococcus aureus, Escherichia coli, Streptococcus viridans, Bacillus subtilis, Monilia albican*
Oxytropis glacialis	Above ground	*Staphylococcus aureus, Escherichia coli, Salmonella limita, Pasteurella multocida, Pasteurella multocida, Streptococcus agalactiae, Bacillus thuringiensis*
Pueraria lobata	Root	*Staphylococcus aureus, Escherichia coli, Dysentery bacilli*
Sophora flavescens	Root	*Staphylococcus aureus, Escherichia coli, α-Hemolytic streptococcus, β-Hemolytic streptococcus, Proteus species*
Sophora alopecuroides	Above ground	*Pythium aphanidermatum, Pseudoperonospora cubensis, Fusarium oxysporium, Alternaria solani, Pseudomonas syringae, Xanthomonas vesicatoria*
Glycine	Fruit	*Fusarium oxyaporum, Mycosphaerella arachidicola*
Cassia obtusifolia	Fruit	*Sclerotinia sclerotiorum, Fusarium, Penicillium digitatum, Ustilaginodiea cirens, Curvularia lunata, Sclerotinia sclerotiorum, Gibberella fujikuroi, Staphylococcus aureus, Escherichia coli, D. cata, Proteus species, Colletotrichum gassypii, Bacillus subtilis, Eupenicillium euglaucum*
Glycine max	Fruit	*Staphylococcus reus, Escherichia coli, Enteropathogenic E. coli, Enterotoxigenic E. coli, Pseudomonas aeruginasa, Samonella typhimurium, Shigenlla flexeneri, Proteus penneri, Klebsiella pneumoniae, Listeria monocytogene, Bacillus cereus, Bacillus subtilis, Monilia albican, Staphylococcus saprophyticus, Staphulocoque blanc, Saccharomyces cercvisiae, Aspergillus niger, Dysentery bacilli*
Leucaena leucocephala	Leaf	*Pseudomonas aeruginosa, Shigella fle xneri, Salmonella typhi,β-Hemolytic streptococcus, Diplococcus penumoniae*

Table 2.1 Plant species and parts with antibacterial activities. (Continued)

Plant name	**Active Part**	**Test microorganism**
Cercis chinensis	Bark	*Staphylococcus aureus, Escherichia coli, Staphylococcus epidermids, Enerococcus, Klebsiella pneumoniae azaenae, Pseudomonas aeruginosa*
Cassia angustifolia	Leaf	*Escherichia coli, Dysentery bacilli, Proteus species,* α *- Hemolytic streptococcus, Monilia albican, Nocardfum asteroides*
Acacia catechu	Steam Leaf	*Staphylococcus aureus, Escherichia coli, Staphylococcus epidermidis, Klebsiella pneumoniae azaenae*

2.4.3 Application of plant open tissue culture

Some researches reported that grape, apple, potato and butterfly orchid were successfully cultured by open tissue culture during rooting period, where the optimizing concentration of antimicrobials in medium was 0.7% (v/v). The roots and plants grew stronger than those by traditional method. One reason was that without autoclaving only a little of the nutritive elements and hormone lost. Another was that the light transmittance of plastic cups were better than glass containers (Chen, 2004; Cui, 2005). Sawant and Tawar used sodium hypochlorite as media sterilant (at total active chlorine concentration 0.002% in the medium) in production of sugarcane plantlets by applying apical meristem culture technique. Use of sodium hypochlorite in place of autoclaving has resulted in reducing considerable amount of electricity cost and ultimately reducing the cost of production of sugarcane plantlets (Sawant and Tawar, 2011); Lu *et al* (2012) used antimicrobial compounds (*Qianxing No.1*) as media sterilant in sugarcane open tissue culture during shooting and multiple phase. Matsumato *et al* (2008) reported that sodium hypochlorite at a concentration of

0.002% was sufficient to prevent microbial growth in the case of banana tissue culture. The plantlets of eucalyptus, banana, pineapple and orchids were successfully cultured by Teixeira *et al* (2006) using sodium hypochlorite in the medium as a disinfectant. They have even reported beneficial effect of the chemical antimicrobials on shoot elongation in eucalyptus, on the number of strawberry shoots, and on the number and length of *Pfaffia glomerata* shoots *in vitro*.

2.4.4 Advantages and disadvantages of plant open tissue culture

Plant open tissue culture technology took benefits from both chemical and botanical antimicrobials as follows: Firstly, antimicrobials with suitable concentration replacing autoclave sterilized media against microbial contamination and establishing fresh shoot cultures from the explants; Secondly, saving electricity expenses and enhancing efficiency of inoculated plantlets in relative aseptic inoculating room rather than in clean bench; Thirdly, reducing the investment of equipments, such as autoclave and clean bench.

On the contrary, the disadvantages of plant open tissue technology might be as follows: the sugar which is contained in culture media affording a capacity of survivalfor microbes. Whether the chemical disinfectants exert any adverse effect on metabolic activities of regenerated shoots need to be investigated using biochemical and/or molecular approaches.

2.5 CO_2 Gas Fertilizer

CO_2 gas fertilizer, an industrial product, releases CO_2 gas slowly when stimulated by artificial light or sun light, and it releases little CO_2 gas in the darkness, the rule of releasing CO_2 gas is suitable for plant photosynthesis (Min *et al.*, 2001).

Although many perfect results were obtained with the application of CO_2 gas fertilizer in greenhouse to produce vegetables, such as cucumber, tomato, watermelon and cabbage and so on (Li, 2007a; Li and Zhou, 2004; Li, 2009; Ma and Wang, 2003). However, there is no report of CO_2 gas fertilizer for sugarcane micropropagation.

There are many research results demonstrated that high atmospheric CO_2 concentration led to increases in photosynthetic rate, whole-plant growth, and water use efficiency (WUE) on many plants. It also decreased stomata conductance and transpiration rate (Kimball *et al.,* 2002; Rogers and Dahlman, 1993; Rogers *et al.,* 1999; Woodward *et al.,* 1991). The plant responses to a high CO_2 level are different, depending on their growth conditions and environmental factors such as tropospheric ozone, temperature, water, irradiance, nitrogen and potassium nutrition levels (Bauer *et al.,* 2001, Ceulemans and Mousseau, 1994; Heaton *et al.,* 2004; Olszyk and Wise. 1997; Silberbush *et al.,* 2003; Torbert *et al.,* 2004). As a new source of carbon for seedling, it is very necessary to research the CO_2 gas fertilizer for its application and benefits in plant tissue culture technology.

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CHAPTER III

SELECTION AND TESTING OF BROAD-SPECTRUM ANTIMICROBIAL COMPOUNDS FOR INHIBITION OF MICROBIAL CONTAMINATION IN SUGARCANE TISSUE CULTURE MEDIA

3.1 Abstract

The objective of this study was to obtain broad-spectrum antimicrobial compounds for inhibition of microbial contamination in plant tissue culture medium. Five common fungus species (*Mucor* sp, *Alternaria* sp, *Penicillium* sp, *Aspergillus flavus* and *Rhizopus* sp) and one bacterial species *Bacillus* sp were isolated from contaminated multiple media in conventional sugarcane tissue culture with the contamination rate about 10.0% and employed as test microbes in this study. Two chemicals (carbendazim and Yi Peilong) and two botanical compounds (nicotine and garlic extraction) were evaluated using the completely randomized design. None of the single compounds was able to inhibit growth of the test microbes. Consequently, an L9 (3^4) orthogonal design was used to study the synergistic effect of chemical and botanical antimicrobial compounds. As a result, broad-spectrum antimicrobial formulation (Qianxing No.1) was developed consisting of nicotine 40.0 mg/L, 57.1% garlic extract 80.0 µl/L, a.i carbendazim 48.0 mg/L and 14.5mg/L Yi Peilong (equal

Yi Peilong (equal 0.00145% active chlorine). These results suggested that medium comprising combined Qianxing No.1 allowed transferring of explants outside the aseptic chamber and provided an effective low-cost option comparing to the traditional practices.

3.2 Introduction

Plant tissue culture technology is a popular method to produce plantlets in a short period. It allows rapid reproduction of genetically disease-free plants that are identical to their origins. However, traditional plant tissue culture technique is a highly sophisticated and expensive technology with some constraints. The cost of production per plant is generally very high, which depends on the production capacity of the laboratory, number of working staff, consumables and electricity expenses. To maintain disinfection and sterilization of the culture media, autoclaving is generally performed which consumes large quantity of electricity (Ahloowalia and Savangikar, 2004; George and Manuel, 2013).

To reduce the cost of plantlets, numerous studies have been performed to test a number of antimicrobial compounds as follows. Bacterial and fungal contamination rates were 0.0% and the highest seedling survival rate was 60.0% when the concentration of carbendazim was 60.0 mg/L in Chinese rose tissue culture (Chen and Wu, 2014). Yi Peilong (contained 0.01% active chlorine) as a novel long-acting broad-spectrum fungicide can prevent contaminate in tissue culture media and it was able to enter cell and interfere DNA duplication, or disconnecting the bond of key protein of microorganism (Jiang *et al.,* 2011; Zhang, 2009). *In vitro* conservation of sweet potato resources, when supplemented 20-25mg/L Yi Peilong in culture media, microbial

contaminated sweet potato could be rescued at 80.0% (Zhou *et al.,* 2008). In *Hevea brasiliensis* root culture, the contamination rate of explants from 80.77% decreased to 44.59% when supplemented with 1.0g/L carbendazim and 0.1%(v/v) Yi Peilong in culture medium (Jiang *et al.,* 2011). Sawant and Tawar used sodium hypochlorite as media sterilant in sugarcane tissue culture, they found that active chlorine concentration of 0.002% in the medium was effective in complete sterilization of the medium (Sawant and Tawar, 2011). But Cui (2005) noted that the application of chemical antimicrobials might cause plantlets mutation, even though they might have efficient inhibition for microbes in the medium (Cui, 2005). Other researchers reported that botanical extracts (such as garlic, nicotine, some activates ingredients of Chinese herbal medicine, etc.) not only had good performance on most microbes, but also were more friendly with plantlets growth and development *in vitro* condition. For example, garlic has been used as a medicine since ancient times and has long been known to have antibacterial, antifungal and antiviral properties (Aala *et al.,* 2014; Bakri and Douglas, 2005; Harris *et al.,* 2001; Weiguo *et al.,* 2004). Pure garlic allicin (6.25 µg/ml and 12.5 µg/ml) was highly efficient in inhibition of the growth of fungal hyphal cells (Aala *et al.,* 2014). Nicotine was very effective against bacterial, especially those that causes disease, like the *Staphylococcus*. It can dissolve cell wall of *Micrococcus* at the concentration of 1-5% in 120 minutes. Its efficiency of inhibiting microbes could be as high as 89.9% (Hu, 2009). Growth of *Streptococcus mutants* cells was significantly repressed by 2.0-8.0 mg/ml of nicotine (Huang *et al.,* 2012).

To minimize the expense of plantlet decontamination, the application of antimicrobial compounds would explore in the culture medium instead of autoclaving to eliminate *in vitro* contamination.

3.3 Materials and Methods

3.3.1 Isolation of fungi and bacteria

For effective isolations to be carried out according to Choi *et al* (1999) described. To single out pure species of fungi and bacteria from contaminated media, we used dissecting needle (or extra fine forceps) picked out of bacterial plaques or hypha from contaminated sugarcane tissue culture bottle with the contamination rate about 10%, then inoculated them in culture dishes which was full with sterilized MS media , one inoculated point per culture dish. All the culture dishes were inoculated in the growth chamber at 25°C. Microscopic examination was used to identify them at a genus level by their morphology before further experiment.

3.3.2 Extraction of active component from tobacco and garlic

3.3.2.1 Extracted nicotine solution

Two hundred grams of dry tobacco (*Nicotiana tabacum* L. White Burley) leaves powder were transferred to a flask and soaked for 5 hours in 500.0 ml NaOH (0.3 mol/L) at 70°C. Subsequently the extract solution was filtered into another flask and added to 40.0 ml of the compound solution containing isopyknic dilute H_2SO_4, Dialkenyl succinimide (4%), DL-2-Octanol (7%), and sulfonated kerosene (89%). The effect then was stirred for 10 minutes and let it stand for stratification. Finally, the bottom liquid portion was drawn out from the flask and the nicotine concentration was quantified by spectrophotometric method, and stored in refrigerator at 4°C until use **(**Al-Tamrah, 1999; Hu, 2009; Palic *et al.,* 2002; Stojanovic *et al.,* 2000).

3.3.2.2 Extracted garlic solution

Seventy grams of fresh garlic cloves (*Allium sativum*) were mashed and blended in 35 ml distilled water, centrifuged, and the supernatant was filtered through a10 kDa cut-off dialysis membrane (Flowgen, UK) and sterilized by membrane filtration (0.45 μm). By subtracting the weight of the insoluble material from the weight of the original cloves, final concentration of the garlic extract in solution was determined to be 57.1% (w/v) **(**Bakri and Douglas, 2005).

3.3.3 Antimicrobial chemicals

1) Carbendazim (80.0% WP) was provided by Qingdao Kai Xing Chemical Industry Co. Ltd., China.

2) Yi Peilong (contained 0.01% active chlorine) was provided by Shanghai Yuhan Bioscience Technology Co. Ltd, China. As a broad-spectrum chemical antimicrobials, the principal component of is sodium hypochlorite.

3.3.4 Sugarcane explants

The axillary buds of sugarcane (*Saccharum* spp) variety *Qiantang 5* were used as explants and obtained by the following steps:

Step 1: Sugarcane stems surface sterilizing. During September 2011, the mature sugarcanes were obtained from the farm of Guizhou Institute of Subtropical Crops., China. The mature sugarcanes were cut into short stems, each with two germination buds. Then the stems were washed with running water to clear away the wax layers and the mildew stains on the surfaces and sterilized with 0.1% carbendazim (80% WP) solution for 30 minutes. After that, they were washed two to three times with running tap water to get rid of the traces of carbendazim.

Step 2: Elimination of sugarcane RSD by heat water treatment. Immerse washed sugarcane stems into constant temperature water bath at 52-53°C for 30 min. Keep stirring to prevent local overheating, because high temperature (over 55°C) will kill the sugarcane buds **(**Benda and Ricaud, 1978; Johnson and Tyagi, 2011).

Step 3: Fostering sugarcane axillary buds. Cover the sugarcane stems with sterilized substrate (peat), just bellows the buds. Cover the buds with moist sterile filter paper or newspaper and culture the cuttings under constant temperature of 38°C and 12h photoperiod conditions 10 to 15 days. Spray water twice per day to keep the substrate and newspaper wet until the buds grow one or two leaves.

Step 4: The axillary buds were obtained from germinated stems. These axillary buds were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) solution and were washed at least three times using sterile distilled water.

3.3.5 Antimicrobial activity test on microbes and explants

The basal MS medium containing 2.5 mg/L 6-BA, pH 5.8 was used as the test medium. The test compounds at different concentrations as indicated in Table 3.1 were incorporated in autoclaved method basal medium before pouring into culture plates or bottles. The experiment was conducted to test each compound individually using a completely randomized design (CRD) in 3 replicated plates (replications) for each test microbe species inoculated at 4 points per plate. For the sugarcane explants, the test was done only on the antimicrobial chemicals which were incorporated into autoclaved basal medium in culture bottles. Two explants were transferred on to the medium in 10 replicated bottles for each concentration of the compounds.

Table 3.1 Concentrations of antimicrobial compounds tested for the activity on test microbes and sugarcane explants.

Test compounds	Concentrations	Concentration unit
Nicotine	0, 10, 20, 30, 40, 50, 60, 70, 80 and 90	mg/L
Garlic extract (57.1%)	0, 10, 20, 30, 40, 50, 60, 70, 80 and 90	µl/L
Carbendazim (80% WP)	0, 50, 55, 60 and 65	mg/L
Yi Peilong (0.29%)	0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7	%(v/v)

3.3.6 Optimized concentration of antimicrobial compounds for microbe inhibition

The carbendazim, Yi Peilong, nicotine and garlic extraction candidates in 3.3.5 were retested using a $L_9(3^4)$ orthogonal design to optimize their best combinations for microbe inhibition in the basal MS medium as shown in Table 3.2. Among the three concentrations, the middle is the best one selected from results of the experiment 3.3.5, the other two are one level lower or higher than that of the best one. After $L_9(3^4)$ orthogonal design experiment had been done, the best combinations of four compounds was selected and stored 200X stock solution at 4℃ for next research. All the species of isolated microbes were inoculated in same plates. Each treatment has three replicated plates.

But before starting the next research, a single factor CRD experiment with 3 replications was necessary to retest the excellent inhibition of antimicrobial compounds

on microbes. The antimicrobial compounds (200X) with different concentrations at 0.0, 0.1, 0.3, 0.5, 0.7, 0.9, 1.1, 1.3, 1.5, 1.7, 1.9 and 2.1% (v/v) were supplemented in non autoclaved basal MS medium.

Table 3.2 L_9 (3^4) orthogonal design for optimization of antimicrobial component

TRT	Nicotine	Garlic Extraction	Carbendazim	Yi Peilong
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	2	1	2	3
5	2	2	3	1
6	2	3	1	2
7	3	1	3	2
8	3	2	1	3
9	3	3	2	1

3.3.7 Data collection and analysis

The plates and bottles were incubated at 25±2℃, 12h photoperiod, and observed daily for the sign of growth for 15 days. Data on survival percentage of each test organisms and explants survival percentage were collected at 15 days after the incubation and subjected to statistical analysis (F test, 0.05) by SPSS 19.0 software and differences of treatment means were compared by Duncan's test (0.05) **(**Hoshmand, 2006; Rocha *et al.*, 2013).

3.4 Results and Discussion

3.4.1 Species of microbes in contaminated media

By morphologically observing under a microscope and biosystematics analyzing, five species of fungi were identified in the contaminated media, including *Mucor* sp **(**Abbas *et al.,* 2002; Collins *et al.,* 1993), *Alternaria* sp **(**Pryor and Michailides, 2002), *Penicillium* sp **(**Amanullah *et al.,* 2000; Pazouki and Panda, 2000), *Aspergillus flavus* **(**Amanullah *et al.,* 2000) and *Rhizopus* sp **(**Du *et al.,* 2003; Teng *et al.,* 2009). Only one species of bacteria, a *Bacillus* sp **(**Hyde *et al.,* 2006). These isolated microbes were employed as test microbes in next study.

3.4.2 Effect of antimicrobial activity on microbes and explants

3.4.2.1 Antimicrobial activity of nicotine extracts on *Bacillus* sp

Nicotine is an alkaloid present in tobacco and accounts for about 3-4% of the dry tobacco weight. The maximum account is about 6.0% in tobacco variety of *White Burley* **(**Li, 2007b). Using the extraction and spectrophotometric methods described by Al-Tamrah and Hu **(**Al-Tamrah, 1999; Hu, 2009), the total 8.0 g of nicotine in 100.0 ml extract solution (0.08 g/ml) was obtained from 200.0 g of dry tobacco leaves. As shown in Figure 3.1, nicotine could inhibit growth of *Bacillus* sp almost completely when the concentration achieved 50.0 mg/L. The result of this study was supported by that of Hu (2009) who reported that when the nicotine concentration was 10.0~50.0 mg/L for 120 min, the efficiency of nicotine to disinfect bacteria (including *Bacillus* sp and *Staphylococcus* sp) was as high as 89.9% .

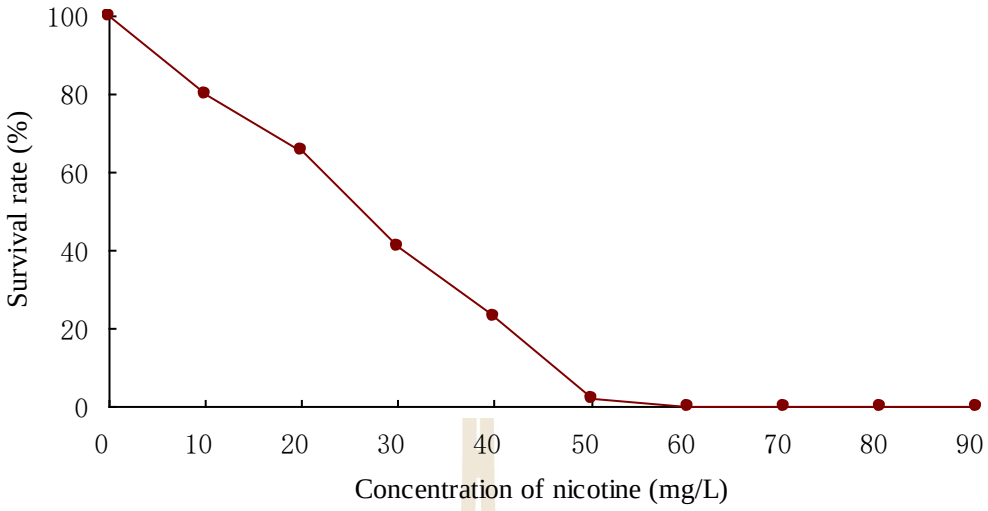

Figure 3.1 Effect of nicotine on survival of *Bacillus* sp at 25°C at the 15th day after inoculation.

3.4.2.2 Antimicrobial activity of garlic extract on fungi

Garlic (*Allium sativum*) has been used as a medicine since ancient times and has long been known to have antifungal and antiviral properties (Cavallito and Bailey, 1944). The main antimicrobial constituent of garlic has been identified as the oxygenated sulphur compound, thio-2-propene-1-sulfinic acid S-allyl ester, which is usually referred to as allicin (Harris *et al.*, 2001). Figure 3.2 shows that garlic extraction (at concentration of 80.0 µl/L) inhibited *Mucor* sp growth by about 99.0%, but had weak effect on *Penicillium sp* and *Aspergillus flavus*, and failed to inhibit *Alternaria* sp and *Rhizopus* sp at added concentration levels. Aala *et al* (2014) found that pure garlic allicin (6.25 µg/ml and 12.5 µg/ml) was more efficient in inhibiting growth of *Trichophyton rubrum* hyphal cells. But in our research garlic extract was chosen instead of pure garlic allicin, because garlic extract was easier to be obtained than the pure garlic allicin, hence, could reduce the cost of plantlet production.

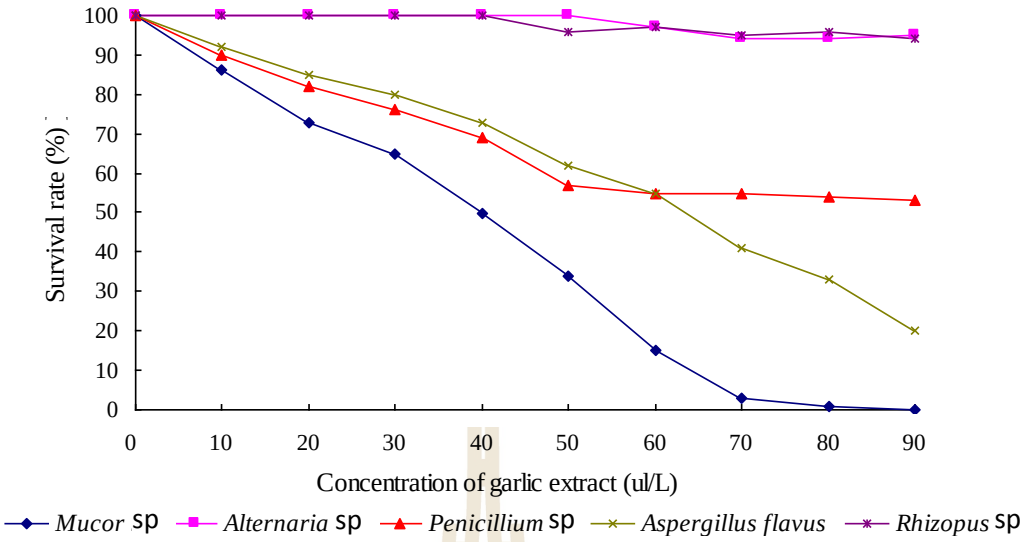

Figure 3.2 Effect of garlic extract on survival of fungi at 25℃ at the 15th day after inoculation.

3.4.2.3 Antimicrobial activity of carbendazim on microbes and explants

Carbendazim is a metabolite of benomyl and widely used broad-spectrum benzimidazole fungicide **(**Li *et al.*, 2014; Nallathambi *et al.*, 2009; Yu *et al.*, 2011). Figure 3.3 shows that the best result was obtained at a carbendazim (80% WP) concentration of 60.0 mg/L, where maximum 51% survival rate of explants were observed. At this condition, *Alternaria* sp and *Penicillium* sp were 100% inhibited. The results are in agreement with the recent observation of Chen and Wu (2014), who reported that microbes contamination rates were 0.0% and seedling survival rate was up to 60% when the concentration of carbendazim (80%WP) was 60 mg/L in Chinese rose tissue culture. On the contrary, carbendazim (80%WP) failed to inhibit *Bacillus* sp and had only weak effect on *Aspergillus flavus*, *Rhizopus* sp and *Mucor* sp.

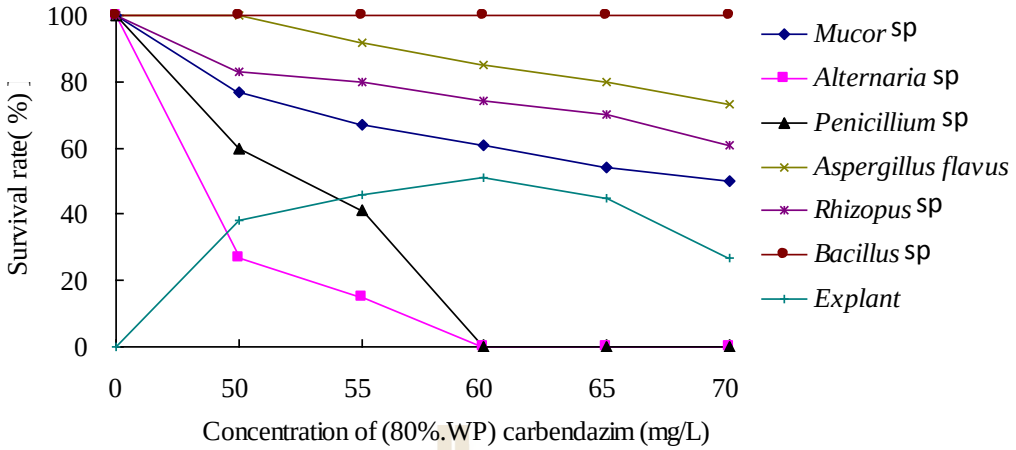

Figure 3.3 Effect of carbendazim on survival of microbes and explants at 25°C on the 15th day after inoculation.

3.4.2.4 Antimicrobial activity of Yi Peilong on microbes and explants

As indicated in Figure 3.4 that Yi Peilong (0.29%) might be able to disinfect all the isolated microbes at the concentration beyond 0.7 %, but we must keep in mind that the survival rate of explants are more important than the microbes inhibition. Therefore, the best alternative concentration of Yi Peilong should be 0.5% (equal 14.5mg/L Yi Peilong or 0.00145% active chlorine), where the maximum 85.0% survival rate of explants was obtained. At this concentration, it inhibited *Penicillium* sp, *Aspergillus flavus* and *Rhizopus* sp 100% at the 15th day after inoculation. With regard to the activity concentration of Yi Peilong 0.5%, our result is different from the research of Jiang *et al* (2011) who reported that in *hevea brasiliensis* root culture, the contamination rate of explants decreased from 80.77% to 44.59% when supplemented with 1.0 g/L carbendazim (80% WP) and 0.1% Yi Peilong in culture medium. It may be because of the different explants having different microbial contaminations.

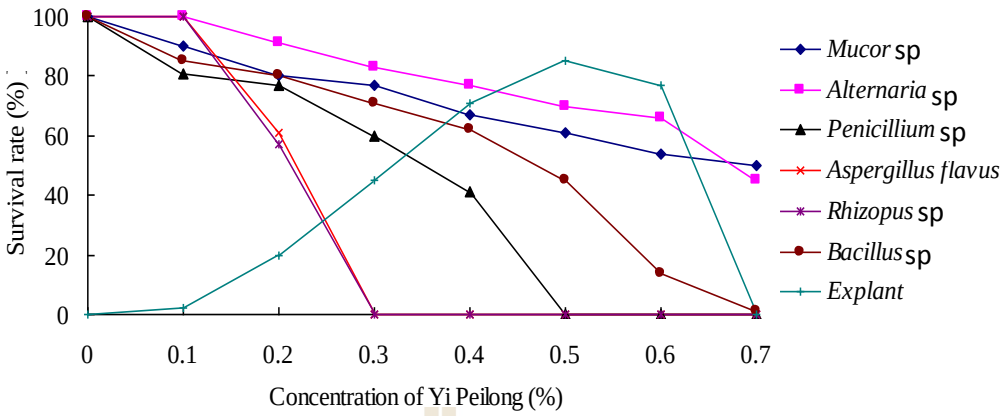

Figure 3.4 Effect of Yi Peilong on survival of microbes and explants at 25°C on the 15th day after inoculation.

3.4.3 Optimized concentration of antimicrobial compounds for microbe inhibition

The aforesaid single factor experiment's results indicated that none of the single antimicrobial compounds (botanical or chemical) was able to prevent microbial contamination up to the desired extent. So, it was worthwhile to combine all of them by an L_9 (3^4) orthogonal design experiment. The best inhibited concentration of single antimicrobials obtained from prelim results. Based on the best inhibited concentration as middle one, we could determine the three concentration levels of each factor: nicotine 40, 50 and 60 mg/L, garlic extraction 70, 80 and 90 µl/L, carbendazim (80% WP) 55, 60 and 65 mg/L and Yi Peilong 0.4, 0.5 and 0.6%. Table 3.3 showed that the best combination was in the TRT-3 (third treatment), comprised nicotine 50 mg/L, garlic extraction 90 µl/L, carbendazim (80% WP) 65 mg/L and Yi Peilong 0.6% respectively. But previous test results (Figures 3.3 and 3.4) indicated that high

concentration of antimicrobial chemicals would cause more harm on the growth of explants as compared to the lower one. Moreover, high concentration of antimicrobial chemicals could not reduce the cost of plant tissue culture and the difference between TRT-2 and TRT-3 was only 1.0%. Therefore, the final concentration of antimicrobial compounds that would be further employed were that of TRT-2 comprising nicotine 40 mg/L, garlic extract 80 µl/L, carbendazim (80% WP) 60 mg/L and Yi Peilong 0.5% respectively. The combination was named as Qianxing No.1.

After mentioned above described, we can see that three best inhibited concentration of single antimicrobials in L_9 (3^4) orthogonal design experiment were the best one in single factor experiments, except for nicotine. Moreover, a synergistic effect between nicotine and other three antimicrobials was observed from Figure 3.1 and 3.5, because the *Bacillus* sp contamination was 23% under the single factor concentration of nicotine 40 mg/L, on the contrary, *Bacillus* sp contamination decreased to 5% when it combined with garlic extraction 80 µl/L, carbendazim 60 mg/L and Yi Peilong 0.5%.

Table 3.3 Microbe survival percentage on MS basal medium supplemented with different combinations of antimicrobial compounds.

TRT	Nicotine (mg/L)	Garlic (µl/L)	Carbendazim (mg/L)	Yi Peilong （%）	Microbe survival (%)
1	40	70	55	0.4	82.00 a
2	40	80	60	0.5	5.00 g
3	40	90	65	0.6	4.00 h
4	50	70	60	0.6	26.17 f
5	50	80	65	0.4	34.17 e
6	50	90	55	0.5	63.17 b
7	60	70	65	0.5	44.33 d
8	60	80	55	0.6	57.33 c
9	60	90	60	0.4	61.83 b

Note : Means followed by the same small letters in the column do not differ by Duncan's test ($P<0.05$), and the CV value was 1.66% in this study. Percentage of contamination rate was transformed to arc sin $(x/100)^{1/2}$ for the analysis of variance.

Table 3.3 indicated the second combination of nicotine 40mg/L, garlic extract 80µl/L, carbendazim (80% WP) 60mg/L and Yi Peilong 0.5% could control the microbial contamination at 5.0%. Then one liter Qianxing No.1 (200X) stock solution was made of 100.0ml nicotine extract from 200.0g dry tobacco leaves, 16.0ml garlic extraction from 70.0g fresh garlic, 2.9g Yi Peilong, 12.0g carbendazim (80% WP) and 884.0ml double distill water.

As shown in Figure (3.5 and 3.6), most of isolated microbes were inhibited completely on non-autoclaved media with the concentration of Qianxing No.1 at 0.5%, except that *Bacillus* sp contamination rate still reached about 5.0%, but this contamination rate was less than the acceptable contamination level of 10.0% in plant open tissue culture which was suggested by Cui *et al* **(**Cui 2005; Cui *et al.*, 2004). Depending on the view of high concentration of antimicrobial compounds would be harmful to the growth and development of plantlets *in vitro*, the concentration of Qianxing No.1 at 0.5% would be the best choice to apply in open tissue culture.

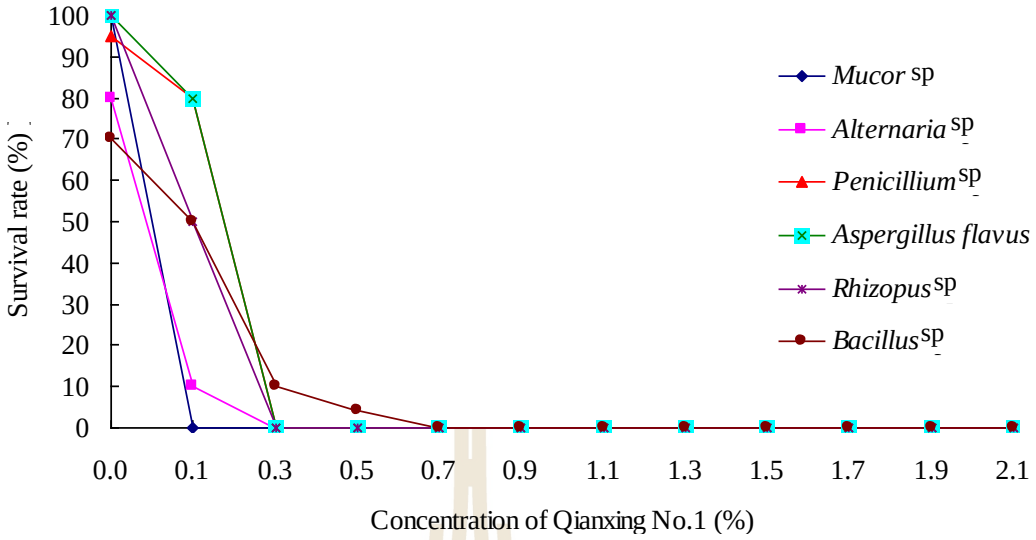

Figure 3.5 Effect of Qianxing No.1 on survival of microbes at 25°C on the 15th day after inoculation.

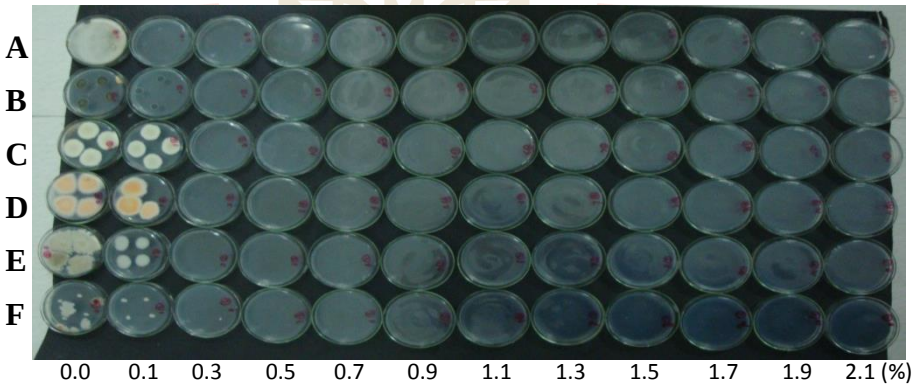

Figure 3.6 Effect of Qianxing No.1 on survival of microbes at 25°C on the 15th day after inoculation. A: *Mucor* sp, B: *Alternaria* sp, C: *Penicillium* sp, D: *Aspergillus flavus*, E: *Rhizopus* sp and F: *Bacillus* sp.

3.5 Conclusion

In this section, five common fungus species (*Mucor* sp, *Alternaria* sp, *Penicillium* sp, *Aspergillus flavus* and *Rhizopus* sp) and one bacterium species *Bacillus* sp were isolated from laboratory environment and employed as test microbes in subsequent research. None of nicotine, garlic extract, carbendazim (80% WP) and Yi Peilong was able to prevent all these isolated microbial contamination and achieve the desired level. Consequently, four antimicrobials were combined to test the inhibited activity on microbes. The combination of nicotine 40 mg/L, garlic extraction 80 µl/L, carbendazim (80% WP) 60 mg/L and 14.5mg/L Yi Peilong (equal 0.00145% active chlorine) has perfect inhibited effects on microbes. Then, a broad-spectrum antimicrobial compounds (Qianxing No.1) was successfully developed. As a result, most of isolated microbes were inhibited completely with the 0.5% concentration of 200X Qianxing No.1, excluding *Bacillus* sp contamination rate still reached about 5.0% which was under the acceptable contamination level of 10.0% in plant open tissue culture as suggested by Cui **(**2005) and Cui *et al* (2004). These results suggested that our novel strategies might contribute to the information of method for controlling microbial contamination in plant open tissue culture.

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CHAPTER IV

THE EFFECTS OF ANTIMICROBIAL COMPOUNDS ON THE GROWTH AND DEVELOPMENT OF SUGARCANE EXPLANTS IN OPEN TISSUE CULTURE

4.1 Abstract

The effects of Qianxing No.1 on the growth and development of sugarcane explants in open tissue culture was investigated. The axillary buds of sugarcane variety *Qiantang* No.5 were used as experimental materials. Different concentrations of Qianxing No.1 were added in the media to determine the optimal concentrations for inducing phase and proliferation phase in sugarcane open tissue culture. The results showed that 200X Qianxing No.1 at 0.5% (v/v) could effectively inhibit the microbial contamination at 6.67% with the 80% survival rate of sugarcane explants, and the propagation efficiency of sugarcane axillary bud of 3.10. The application of Qianxing No.1 was able to reduce the equipment and electricity costs, as well as improve the production efficiency by facilitating the whole culture process. The results suggested that sterilization of the medium with Qianxing No.1 and explants transferring in a less aseptic condition, rather than in laminar chamber, can be a low cost and effective option during the inducing and proliferate phases of sugarcane explants when compared to conventional plant tissue culture.

4.2 Introduction

The crossbreeding of sugarcane takes as long as 8 to 10 years. In production, sugarcane stem is usually used for cloning. However, long-term asexual reproduction will aggravate the sugarcane viral diseases, fungal diseases and bacterial diseases, leading to the genetic depression of sugarcane (Liu *et al.*, 2009). Applying tissue culture in sugarcane production is one of the effective ways to solve these problems. Currently, 90% of the sugarcane seedlings in Brazil, Cuba and other countries are healthy and virus-free. In China, the researches and application of tissue cultured sugarcane seedlings have been carried out in Guangxi, Yunnan, Hainan, Fujian and other sugarcane growing areas. It was reported that the application of healthy virus-free sugarcane seedlings could increase sugarcane yield by 20-40%, and increase sucrose content to 0.5% (Dan *et al.*, 2011).

Plant tissue culture technology is a popular method to produce plantlets in a short period. It allows rapid reproduction of disease-free plants which are genetically identical to their origin. However, traditional plant tissue culture technique is a highly sophisticated and expensive technology with some constraints. The cost of production per plant is generally very high, which depends on the production capacity of the laboratory, number of working staff, consumables and electricity expenses. Especially for maintaining disinfection and sterilization of the culture media, autoclaving is generally performed which consumes large quantity of electricity **(**Ahloowalia and Savangikar, 2004; George and Manuel, 2013). To reduce the cost of plantlets, numerous studies have been performed to test a lot of antimicrobials as follows. Cui and Chen (2005) had successfully cultured the plantlets of grape, apple, potato and butterfly orchid by open tissue culture in rooting period, where the optimizing

concentration of antimicrobials in medium was 0.7%. The roots and plants grew stronger than those by traditional tissue culture. One reason was that non autoclaved media contributed a little of loss on nutritive elements and hormone. Another was that the light transmittance of plastic cups was better than glass container **(**Chen, 2004; Cui, 2005). Matsumato *et al* (2008) reported that sodium hypochlorite at a concentration of 0.002% was sufficient to prevent microbial growth in the case of banana tissue culture. Sawant and Tawar (2011) used sodium hypochlorite as media sterilant in sugarcane tissue culture; they found that active chlorine concentration of 0.002% in the medium was effective in complete sterilization of the medium.

To minimize the expense of electricity and labors, it was thought worthwhile to study the application of Qianxing No.1 instead of autoclaving to eliminate contamination in culture media.

4.3 Materials and Methods

4.3.1 Sugarcane explants

The axillary buds of sugarcane variety *Qiantang 5* used as explants were obtained by the methods of section 3.3.4. Cut off the axillary buds germinated from stems, remove the axillary buds top leaves and strip off root sheath leaves. And proceed with the following experiments:

For conventional micropropagation (CK1): axillary buds were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) solution for 15 min under the aseptic chamber. After disinfection, the buds were washed 1-2 times with sterile water, 5-10 min each time. Subsequently, they were placed on sterile filter paper to adsorb the surface water.

For open tissue culture: the buds were wiped with 70.0% alcohol, then soaked in 0.4% (*v*/*v*) 200X Qianxing No.1 for 15 min. Finally, one or two layers of leaf sheathes were carefully peeled off by forceps and scalpel, retaining the top growing point about 0.5-1.0 cm, the browning parts were cut away of buds to get the explants for further experiment. All steps were done in an open condition outside the aseptic chamber.

4.3.2 Antimicrobial compounds

As described in chapter III, one liter antimicrobial compounds (Qianxing No.1) 200X stock solution was made containg 100.0ml nicotine extract from 200.0g dry tobacco leaves, 16.0ml garlic extraction from 70.0g fresh garlic, 2.9g Yi Peilong, 12.0g carbendazim (80% WP) and 884.0ml double distilled water, stored in refrigerator at 4°C until use.

4.3.3 Disinfection of transferring room and culture tools

First, an ozone apparatus (Type: HT-CY -30, which was provided by Jinan Haotian Technology Development Co., LTD, China) was employed to disinfect transferring room for 2h in the night. Then the transferring room was treated with 75% ethanol for falling dust and was irradiated with UV light for 15-20 min. The disinfection of culture tools, including flasks, metal discs, forceps and scalpels, were soaked in 1% (*v*/*v*) Qianxing No.1 (200X) for 12h, and then drained for use.

4.3.4 Culture media and explants transferring methods

To test the effects of Qianxing No.1 on the growth and development of explants and their efficacy in controlling contamination under the active working condition, an experiment was conducted in CRD with 12 treatments, three replications. The basal inducing MS medium contained 6-BA 2.5 mg/L; basal

proliferation MS medium contained 1.0mg/L 6-BA and 0.5mg/L KT. Temperature and photoperiod were maintained at 25±2ºC and 12 h respectively. The treatments were as follows:

4.3.4.1 Conventional micropropagation (CK1)

During the inducing phase, two axillary buds were transferred on autoclaved basal inducing medium in each flask for 10 flasks. Subsequently, they were subcultured onto the basal proliferation medium after the differentiation. The process was performed in the aseptic transferring chamber.

4.3.4.2 Open tissue culture

The explants were transferred onto non-autoclaved inducing and proliferation basal MS media supplemented with Qianxing No.1 (200X) with the concentration of 0.0(CK2), 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0% in the disinfected transferring room, outside the aseptic chamber. The non-autoclaved medium pH was adjusted to 5.8 after Qianxing No.1 was added in the medium. The numbers of experiment units are same as CK1.

4.3.5 Data collection and analysis

Percentage of both microbial contamination and explants survival were observed after 15 days for inducing phase and 20 days for proliferation phase respectively, as well as browning and propagation efficiency of the explants. Contamination (%) = number of contaminated axillary buds/number of transferred axillary buds; Browning (%) = number of browning axillary buds/number of transferred axillary buds; Surviving (%) = number of survived axillary buds/number of transferred axillary buds; Propagation efficiency=post-transferred buds/transferred buds. Where, some of browning buds with the capability of well growth and

development were classified as survival buds. The buds with height shorter than 2.0 cm were not collected to analysis. The data were statistically analyzed (F test, 0.05) by SPSS 19.0 software and treatment means were compared by Duncan's test (0.05) **(**Hoshmand, 2006; Rocha *et al.,* 2013).

4.4 Results and Discussion

4.4.1 Effect of Qianxing No.1 on sugarcane in inducing sugar medium

As shown in Table 4.1 and Figure 4.1, the contamination rate decreased with the increase of Qianxing No.1 concentrations. When they were greater than or equal to 0.5%, contamination rate ranged among 0.0-6.67%, which was significantly lower than that of the other treatments (P<0.05), including the conventional plant tissue culture treatment (CK1). The browning rate increased with the increase of Qianxing No.1 concentration. When the concentration was 0.3%, the browning rate was close to that of the CK1, but significantly different from that of the 5%concentration (P<0.05). The surviving rate trended to have a parabolic variation, and it reached the peak (80%) when the Qianxing No.1 concentration was 0.5%, and then decreased with the increase of Qianxing No.1 concentration. In overall, although the contamination was slight for the concentration of 0.6%, the browning phenomenon was severe when the concentration of Qianxing No.1 was higher than 0.6%, the survival rate of the explants was low; when the concentration of Qianxing No.1 range between 0.5% and 0.6%, no significant difference among the surviving rates. In reality, low Qianxing No.1 concentration means reduced costs. As CK2 shows, without Qianxing No.1 in medium, all explants couldn't survive because of the 100% microbial contamination.

Therefore, the optimal concentration of 200X Qianxing No.1 in inducing phase of sugarcane variety *Qiantang 5* was set at 0.5%.

Table 4.1 Explant development observed after 15 days *in vitro* inducing medium supplemented with different concentrations of Qianxing No.1 (200X).

TRT	Contamination (%)	Browning (%)	Survival (%)
CK1	56.67 b	43.33 e	43.33 c
CK2	100.00 a	31.67 f	0.00 f
0.1%	91.67 a	25.00 g	15.00 e
0.2%	56.67 b	30.00 f	36.67 d
0.3%	43.33 c	43.33 e	45.00 c
0.4%	18.33 d	50.00 d	70.00 b
0.5%	6.67 e	53.33 c d	80.00 a
0.6%	3.33 f	58.33 c	75.00 a b
0.7%	1.67 f g	75.00 b	0.00 f
0.8%	0.00 g	95.00 a	0.00 f
0.9%	0.00 g	100.00 a	0.00 f
1.0%	0.00 g	100.00 a	0.00 f
CV (%)	11.12	2.81	6.62

Note: Means followed by the same small letters in the column do not differ by Duncan's test ($P<0.05$). Percentage of contamination and survival was transformed to arc sin $(x+0.5)^{1/2}$ for the analysis of variance.

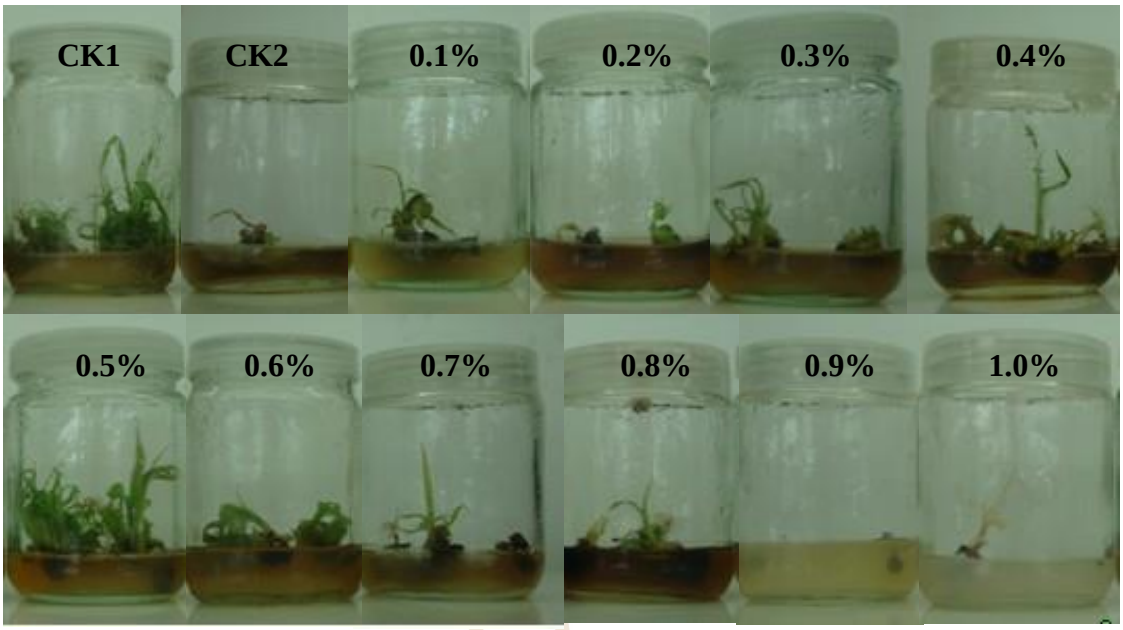

Figure 4.1 Effects of Qianxing No.1 with different concentrations on the growth and development of sugarcane axillary buds during inducing phase 15 days after transferring. CK1 and CK2 were traditional plant tissue culture and Qianxing No.1 free treatment respectively.

4.4.2 Effect of Qianxing No.1 on sugarcane in proliferation sugar medium

As shown in Table 4.2, the contamination rate decreased with the increase of Qianxing No.1 concentrations. When they were greater than 0.4%, the contamination rate ranged was among 0.0-11.0%, which was significantly lower than that of the other treatments, including CK1 treatment (P<0.05). The browning rate increased with the increase of Qianxing No.1 concentrations. When it was 0.4%, the browning rate was not significantly different to that CK1, but different from that of the 0.5% concentration (P<0.05). The propagation efficiency trended to have a parabolica variation, and it reached the peak (3.10) when the Qianxing No.1 concentration was 0.5%, which showed no significant difference to that of the CK1. Considering the

maximizing of propagation efficiency and saving costs, the optimal concentration of 200X Qianxing No.1 in the proliferation culture of sugarcane was also set at 0.5%.

The contamination rate and browning rate of the open tissue culture were significantly higher than that of the conventional culture under the Qianxing No.1 concentration of 0.5%, but the propagation efficiency of the later showed no significance from that of the conventional culture. This might suggest that some components of the Qianxing No.1 inhibited the development of differentiated seedlings in early period, and this inhibition became weaker with the rapidly growth and development of seedlings *in vitro*. This hypothesis was demonstrated by the well growth and development of cluster buds *in vitro* as Figure 4.2 shows, under the concentration of 0.5% Qianxing No.1, the growth and development of sugarcane cluster buds were as good as that of the CK1. Moreover, as CK2 shows, without Qianxing No.1 in medium, all explants couldn't survive after 20 days because of the 100% microbial contamination.

Table 4.2 Explant development observed after 15 days *in vitro* in the proliferation medium supplemented with different concentrations of Qianxing No.1.

TRT	Contamination (%)	Browning (%)	Propagation efficiency
CK1	7.00f	32.33e	3.12a
CK2	100.00a	22.00g	0.00g
0.1%	42.67b	22.33g	0.96f
0.2%	22.33c	20.00g	1.55e
0.3%	15.33d	26.67f	2.07d
0.4%	11.00e	31.00e	2.72b
0.5%	9.33e	45.67d	3.10a
0.6%	5.67f	62.00c	2.60c
0.7%	1.67g	87.33b	2.06d
0.8%	0.67h	100.00a	1.00f
0.9%	0.00i	100.00a	1.00f
1.0%	0.00i	100.00a	1.00f
CV (%)	4.62	3.61	1.13

Note: Means followed by the same small letters in the column do not differ by Duncan's test ($P<0.05$). Percentage of contamination and survival was transformed to arc sin $(x+0.5)^{1/2}$ for the analysis of variance.

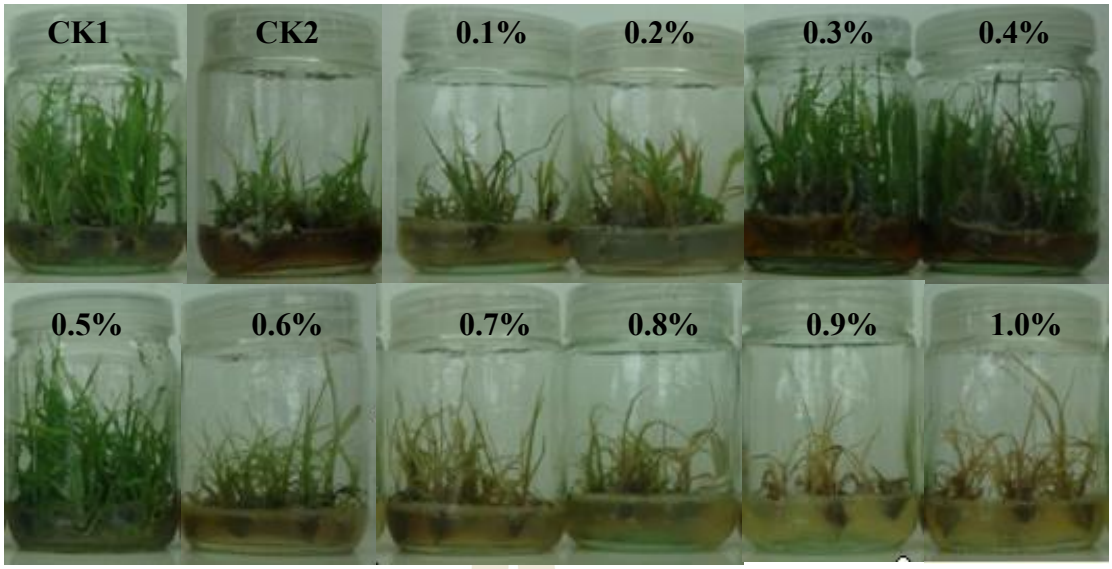

Figure 4.2 Effect of different concentrations of Qianxing No.1 on the growth and development of sugarcane cluster buds during the proliferation phase 15 days after transferring. CK1 and CK2 were traditional plant tissue culture and Qianxing No.1 free treatment respectively.

4.5 Conclusion

Compared with the conventional tissue culture technique for sugarcane, Qianxing No.1 established a new tissue culture system by adding 0.5% of this antimicrobial formulation into the sugarcane inducing and proliferation media. By this system, it could effectively reduce the costs of sugarcane plantlets, because the application of Qianxing No.1 could replace media sterilization by autoclaving. Hence reduce the cost of sterilization. The new transferring method could omit the use of aseptic chamber and save 46% of the total costs used for running the chamber **(**Yan *et al.*, 2007). Besides, the propagation efficiency of conventional tissue culture and open tissue culture are the same. Moreover, the new transferring method simplified and

reduced the requirements for procedure, improving the transferring efficiency. In short, the application of Qianxing No.1 did not only reduce the equipment cost and electricity cost, but also improved the production efficiency and reduced the labor costs.

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CHAPTER V

EFFECT OF THE COMBINATION OF ANTIMICROBIAL COMPOUNDS AND CO_2 GAS FERTILIZER ON THE GROWTH AND DEVELOPMENT OF SUGARCANE EXPLANTS IN SUGAR-FREE MEDIA

5.1 Abstract

The aim of this research was to evaluate the efficacy of application of CO_2 gas fertilizer combining with Qianxing No.1 in sugar-free tissue culture for sugarcane by a completely randomized design of a 3x3 factorial experiment with two factors in three replications. CO_2 gas fertilizer of three dose levels (0.9, 1.0 and 1.1g (equal 1837.6, 2144.0 and 2450.0 ul/L CO_2 gas) in 250ml culture bottle), 200X Qianxing No.1 with three concentration levels (0.4, 0.5 and 0.6 %). Sugarcane (*Qiantang No.5*) explants were evaluated for their growth and development during the multiplication and rooting phases under controlled condition of 25±2ºC and 12h photoperiod. Explants were transferred in an invented CO_2 gas fertilizer culture bottle in outside aseptic chamber. Net photosynthesis was measured by the modified IRGA (LI 6400-XT). The expected results were obtained in the presence of CO_2 gas fertilizer at 1.0 g per culture bottle (equal to 2144.0 µl/L of CO_2 gas) and Qianxing No.1 (200X) concentration of 0.5% respectively, where maximum 3.15 of propagation efficiency and minimum 3.5% contamination rate were achieved in the multiplication phase.

The percentage of survival and contamination, average number of roots, leaves, net photosynthesis and weight per plantlet were 96.67%, 3.5%, 14.7 roots, 7.9 leaves, 20.28 μmol^{-s} and 2.53g respectively in the rooting phase. For production of disease-free plantlets, it was critical that heat treated sugarcane stems (by 52-53℃ water) and disease detection by PCR during the early stage of culture process should be used and performed. These results suggested that the novel strategy of low-cost open tissue culture technology could overcome the defects of sugar-free and open tissue culture technologies by combing their advantages by the application of CO_2 gas fertilizer in sugar-free tissue culture. This strategy offers a low-cost and effective option that is superior to the traditional plant tissue culture.

5.2 Introduction

Sugarcane micropropagation *in vitro* has been routinely used in China in order to produce healthy plantlets from new improved cultivars, which can be more readily available to farmers. However, with the widely use of plant tissue culture technology, some problems are arising as follows. First, excess of sucrose concentration in the media not only might cause cellular dehydration by osmotic gradient, but also lead to higher microbe contaminations **(**Perez *et al.*, 2004). Second, for maintaining disinfection and sterilization of the culture media, autoclaving is generally performed, consuming large quantity of electricity **(**Ahloowalia and Savangikar, 2004; George and Manuel, 2013).

In order to solve these problems, a number of reports have been published on the reduced microbial contamination rate and enhanced growth of plantlets *in vitro* by

using sugar-free technology, increasing photosynthetic photon flux (PPF) and the CO_2 concentration in the vessel. These reports include on carnation culture **(**Kozai and Iwanami, 1988), eucalyptus culture **(**Kirdmanee *et al.*, 1995), potato culture **(**Niu and Kozai, 1997), statice culture **(**Xiao and Kozai, 2006a; Xiao *et al.*, 2011). Furthermore, other group of reports on plant open tissue culture technique has also been published on the reduced cost per plantlet by using antimicrobial compounds instead of autoclaved media against microbial contamination. The application could save electricity expenses and enhance efficiency of plantlet handling in an open condition. These reports include grape, apple, potato and butterfly orchid open tissue culture **(**Chen, 2004; Cui, 2005). Sugarcane micropropagation which was used sodium hypochlorite as media sterilant **(**Sawant and Tawar, 2011).

In spite of the above advantages, there are still some defects of sugar-free tissue culture and open tissue culture. In open tissue culture, antimicrobial compounds were supplemented in non-autoclaved media, although the operation steps could be done outside aseptic chamber, but the sugar contained in media could support growth of microbes. For the sugar-free tissue culture, the whole operation steps have to be conducted inside aseptic chamber and the complicated CO_2 gas applies system is expensive **(**Guan, 2007). These are the reasons why open and sugar-free tissue culture technologies have not been so far widely used.

Plant species are autotrophic. Previous studies displayed an increase in sugarcane photosynthesis grown under elevated CO_2 **(**De *et al.*, 2008; Vu and Allen, 2009a; Vu and Allen, 2009b; Vu *et al.*, 2006a), but most of them could not express such property when cultivated *in vitro*, because of the low CO_2 supply and low gas exchange inside the culture flask **(**Kozai *et al.*, 2005). Guan (2007) reported the

suitable density of CO_2 for seedlings growth was 2000-4000 ul.L^{-1}.

CO_2 gas fertilizer, an industrial product, releases CO_2 gas slowly when stimulated by artificial light or sun light, and it releases little CO_2 gas in the darkness, such a rule of release CO_2 gas is suitable for plant photosynthesis **(**Min *et al.*, 2001). However, there is no reports of CO_2 gas fertilizer application for sugarcane micropropagation, although there have been many successes with the application of CO_2 gas fertilizer in greenhouse to produce vegetables, like cucumber, watermelon, tomato and cabbages **(**Li, 2007a; Li and Zhou, 2004; Li, 2009).

This section was therefore conducted to evaluate the efficacy of combination of Qianxing No.1 and CO_2 gas fertilizer on the supporting of growth and development of sugarcane explants on sugar-free medium.

5.3 Materials and Methods

5.3.1 Sugarcane explants

In this section, the explants were sugarcane cluster buds produced by the open tissue culture in chapter IV.

5.3.2 Carbon dioxide gas fertilizer culture bottle

In sugar-free tissue culture, simply removing sugar from the culture medium without increasing PPF and CO_2 concentration inside the vessel would not promote growth of culture or plantlets **(**Xiao *et al.*, 2011). Insufficient supply of CO_2 during photoperiod will inhibit plantlet photosynthesis as supported by recent studies on myrtle plantlets **(**Lucchesini *et al.*, 2001; Lucchesini *et al.*, 2006). Consequently, numerous studies have shown two options to enhance the CO_2 concentration inside the vessel. First, for small culture vessels, CO_2 gas enhances by natural ventilation

through the gaps of the contact surfaces of the vessel. Natural ventilation is based on diffusion through the air gap between inside and outside air of the vessel or through a gas permeable membrane filter attached on the lip or on the wall of the vessel. Second, CO_2 gas enhances with forced ventilation for large culture vessels. Forced ventilation is more effective in gas exchanges and can be achieved by pumping a particular gas mixture with an air pump into the culture vessel through gas permeable filter disk **(**Kozai *et al.,* 2000; Zobayed *et al.,* 1999). However, with both options, natural ventilation through the gaps of the contact surfaces of the vessel is less effective in gas enrichment and might increase microbial contamination. Besides, in forced ventilation system, growth of plantlets in the vessel was not uniform, with larger plantlets near the air inlet and comparatively smaller plantlets near the air outlet **(**Heo and Kozai, 1999). Moreover, it is critical that both of them required sterilized culture media.

Therefore, our research group successfully designed and applied a Chinese utility model patent (Patent No: ZL 2012 2 0277809.5) **(**Lu *et al.,* 2013), the carbon dioxide gas fertilizer culture bottle was able to overcome those defects mentioned previously. As Figure 5.1 shows, through the hole (A), CO_2 gas fertilizer could be put and kept in a small tray (E) in culture bottle and supplies carbon sources for plantlet photosynthesis in the sugar-free medium (G).

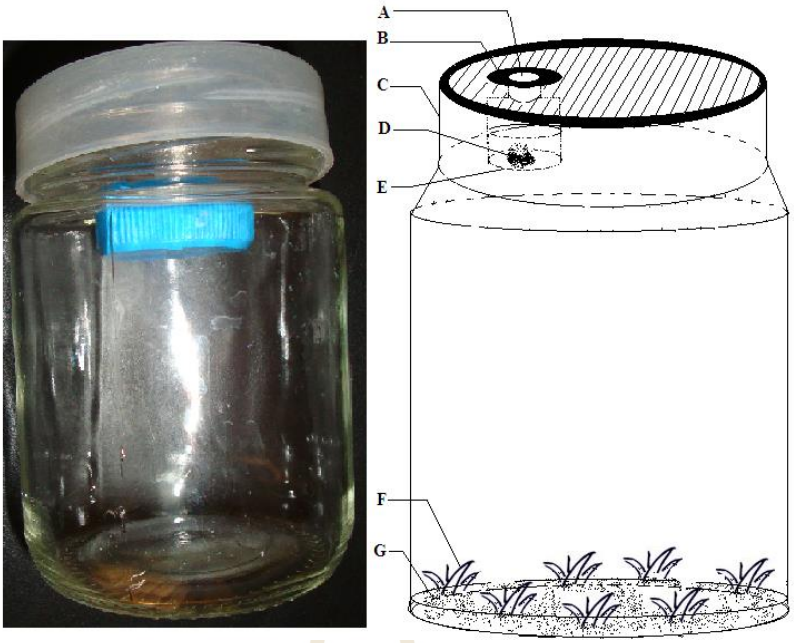


Figure 5.1 Carbon dioxide gas fertilizer culture bottles. A: a hole for putting CO_2 gas fertilizer in a tray. B: a screw cap for closing A and isolating outside microbes. C: a bottle cover. D: CO_2 gas fertilizer in a tray. E: tray; F: plantlets; G: sugar-free medium.

5.3.3 Antimicrobial compounds

As described in chapter Ⅲ, one liter antimicrobial compounds (Qianxing No.1) 200X stock solution was made contained 100.0ml nicotine extract from 200.0g dry tobacco leaves, 16.0ml garlic extraction from 70.0g fresh garlic, 2.9g Yi Peilong, 12.0g carbendazim (80% WP) and 884.0ml double distilled water, stored in refrigerator at 4°C until use.

5.3.4 Determination of CO_2 gas released form CO_2 gas fertilizer

The CO_2 gas fertilizer was provided by Beijing Jin Ling Shuang Environmental Protection Equipment Company Limited., China. Five groups of

cylinders (1000ml and 500ml), (500ml and 250ml), (250ml and 100ml), (100ml and 50ml) and (50ml and 20ml) were used to measure the volume of CO_2 gas released by CO_2 gas fertilizer at 25g, 20g, 15g, 10g and 5g under the tissue culture condition with 25±2℃ (day and night) and 12h photoperiod.

As Figure 5.2 shows, CO_2 gas fertilizer kept in small vessel in the bottom of small cylinder, then the small cylinder was overturned and kept in big cylinder which was filled with water, but there were a high water level in small cylinder and low water level in big cylinder respectively. Under the stimulation of light, CO_2 gas fertilizer which was kept in the small vessel would release CO_2 gas day by day **(**Min *et al.,* 2001). With the increase of CO_2 gas, the scale value would be enlarged from O to E. CO_2 gas volume at the 3^{th} day was A subtract O, CO_2 gas volume at the 6^{th} day was B subtract A. Follow this method, CO_2 gas volume at the 15^{th} day was E subtract D. The data of CO_2 gas volume in cylinder was collected every three days until no CO_2 gas was released and subjected to an analysis by linear regression.

Figure 5.2 A device to measure the volume of released CO_2 gas from CO_2 gas fertilizer. O: scale value at 0^{th} day. A: scale value at 3^{th} day. B: scale value at 6^{th} day. C: scale value at 9^{th} day. D: scale value at 12^{th} day. E: scale value at 15^{th} day.

5.3.5 Detection of RSD and SCMV in sugarcane plantlets

Plant Genomic DNA Kit (DP305), RNAprep Pure Plant Kit (DP432) and TIANScript II RT Kit (KR107) were provided by Tiangen Biothch (Beijing) Co., LTD, China (www.tiangen.com/en). The specific primers Cxx1 (5-CCGAAGTGAG CAGA TTGACC) and Cxx2 (5-ACCCTGTGTTGTTTTCAACG); SCMV-F (5-GAT GCAG GVGCHCAAGGRGG) and SCMV-R (5-GTGCTGCTGCACTCCCAACAG) were provide by Sangon Biothch (Shanghai) Co., LTD, China (www.sangon.com).

In multiple phases, after young leaves were germinated from first genera- tion sugarcane cluster buds which were induced from single bud, taken 100.0 mg fresh leaves to extracted DNA samples by Plant Genomic DNA Kit (DP305), then detect

RSD (*Clavibacter xyli* subsp) by the specific primers Cxx1-Cxx2 which was reported by Pan *et al* (1998). Taken 100.0 mg fresh plantlet leaves to extracte RNA samples by RNAprep Pure Plant Kit (DP432). The plantlet cDNA was obtained by TIANScript II RT Kit (KR107), and then RT-PCR detects SCMV (*Sugarcane mosaic virus*) pathogen from cDNA sample by the specific primers SCMV-F & SCMV-R which was reported by Tang (2006).

Finally, disease-free cluster buds were chosen for next multiple cultures. The same detection method of RSD and SCMV was employed in rooting phases to test disease-free sugarcane plantlets.

5.3.6 Effect of combination of Qianxing No.1 and CO_2 gas fertilizer on the growth and development of sugarcane explants in sugar-free medium

As is well-know that plant species are autotrophic except for the plant buds **(**Xiao and Kozai, 2006a; Xiao *et al.*, 2011). Consequently, the growth and development of sugarcane cluster buds with the capability of autotrophic in culture bottle were studied during the multiplication and rooting phases. The experiment consisted of a completely randomized design, arranged in a 3×3 factorial with three replications. One of the factor was CO_2 gas fertilizer with three doses at (C-0.1)g, C g and (C+0.1)g per culture bottle, where, C is the best dose of CO_2 gas fertilizer which releases CO_2 gas within 15 days in the experiment 5.3.4. Another factor was Qianxing No.1 (200X) with three concentrations at 0.4%, 0.5% and 0.6% (v/v). In a disinfected room, explants were transferred onto Qianxing No.1 contained sugar-free media in carbon dioxide gas culture bottles. Experimental unit consisted of twenty bottles, each bottle containing five explants. The culture medium pH was adjusted to 5.8 after Qianxing No.1 was added into the medium.

5.3.6.1 Combination of Qianxing No.1 and CO_2 gas fertilizer during the multiplication phase of explants in sugar-free medium

The multiplication phase was studied with 20±3mm length explants grown in 250.0 ml flasks containing 40.0 ml of sugar-free MS medium with the addition of 1.0 mg/L 6-BA, 0.5 mg/L KT and 4.0 g/L agar, during the subsequent subcultures for 20 days. Temperature and photoperiod were maintained constant at 25 ±2ºC and 12 h, respectively. The 20-day subculture average data constituted the data for statistical analysis including contamination rate, survival rate and propagation efficiency of explants, and bud number per 100 explants.

5.3.6.2 Combination of Qianxing No.1 and CO_2 gas fertilizer during the rooting phase of sugarcane explants in sugar-free medium

The rooting phase study was conducted with 25-30 mm length buds which was obtained from multiplication phase, grown for just one 25-day period in sugar-free MS medium with the addition of 3.0 mg/L NAA and 4.0 g/L of agar. During this phase, the same growth conditions of light and temperature were used as the multiplication phase. The contamination and survival rate, average number of root, leaf, and height per plantlet were the variables statistically evaluated.

5.3.7 Measurement net photosynthesis of sugarcane plantlets

In terms of photosynthesis measurement, the CO_2 exchange is the most commonly used for building photosynthesis monitoring systems. In this technique, a commercial photosynthesis monitoring systems IRGA (LI-6400XT) was developed and widely used **(**Millan-Almaraz *et al.*, 2009). But the sample chamber of IRGA (LI-6400XT) is too smaller (about 6.0 $cm^{2)}$ to measure the photosynthesis of a whole

plantlet, especially for sugarcane plantlets with long and narrow leaves *in vitro* condition. Consequently, our research group invented a rapid method to measure the net photosynthesis of whole plantlet *in vitro* condition as shown in Figure 5.3.

Standard leaf chamber and IRGA sample chamber of IRGA (LI-6400XT) photosynthesis system were removed from self-trivial handle compartment lid. Then IRGA sample chamber was combined with a homemade leaf chamber by soil mounting plate. The homemade leaf chamber, made of transparent resin material, is a rectangular-shaped container (20cm×10cm×10cm) with a moveable cover on the top to keep the culture bottle. Finally, the IRGA sample chamber was connected with the host of photosynthesis monitoring system IRGA (LI-6400XT) through data lines. To use the modified IRGA (LI-6400XT), uncovered plantlets were put in homemade leaf chamber to rapidly measure the net photosynthesis of the plantlets.

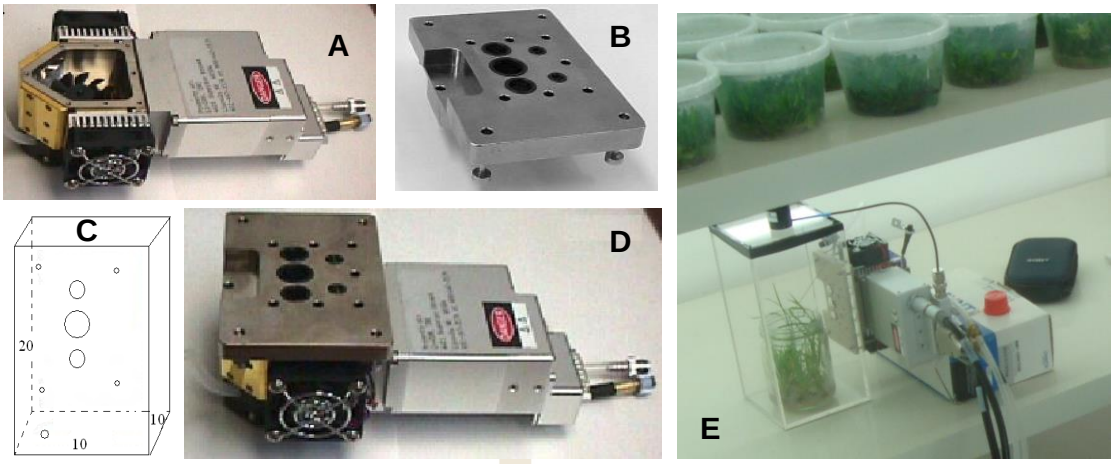


Figure 5.3 A device of rapid measurement of net photosynthesis of a whole plantlet *in vitro*. A: IRGA sample chamber. B: soil mounting plate. C: homemade leaf chamber. D=A+B. C+D=E: modified IRGA (LI-6400XT).

5.3.8 Data collection and analysis

Percentage of contamination, explants survival, browning and propagation efficiency were collected follow the methods of section 4.3.5. Average number of root, leaf, net photosynthesis, weight and height per plantlet were observed after 25 days for rooting phase (20 days for proliferation phase). Where, the leaves which were shorter than 1.5 cm will not be collected. The data were statistically analyzed (F test, 0.05) by SPSS 19.0 software and difference of treatment means were compared by Duncan's test (0.05) **(**Hoshmand, 2006; Rocha *et al.*, 2013).

5.4 Results and Discussion

5.4.1 Rule of CO_2 gas fertilizer releases CO_2 gas

Table 5.1 shows that, with the dose increase of CO_2 gas fertilizer from 5g to 25g, the total volume of released CO_2 gas increased from 3.6 ml to 19.0 ml under the

plant tissue culture condition. Additional, from the Figure 5.4, we can see large amount of CO_2 gas was released during the 3th day to the 6th day, and little CO_2 gas was released during the 12th day to the 15th day.

Table 5.1 Rule of CO_2 gas fertilizer releases CO_2 gas.

CO_2 gas fertilizer	**3thday**	**6th day**	**9th day**	**12th day**	**15thday**	**Total (ml)**
5g	1.5	1.4	0.5	0.2	0.0	3.6
10g	2.5	3.4	1.3	0.3	0.0	7.5
15g	3.0	5.0	2.7	0.5	0.0	11.2
20g	4.0	6.0	4.2	0.8	0.0	15.0
25g	5.0	8.0	5.0	1.0	0.0	19.0

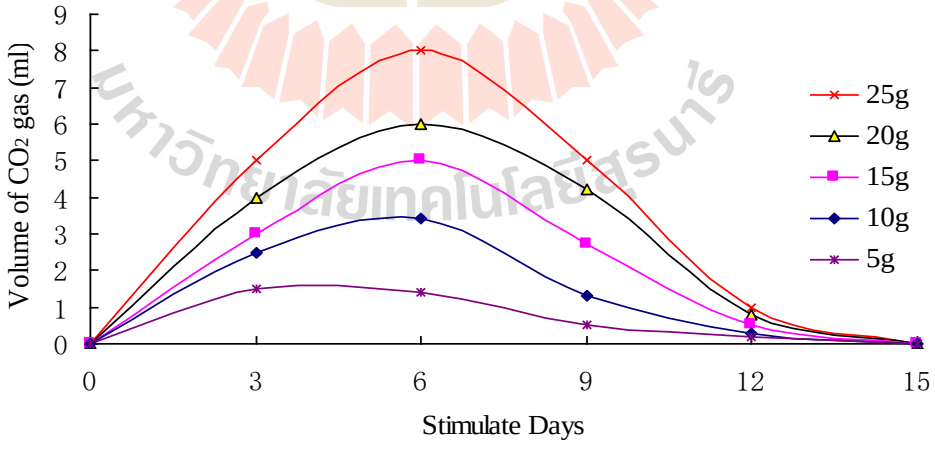
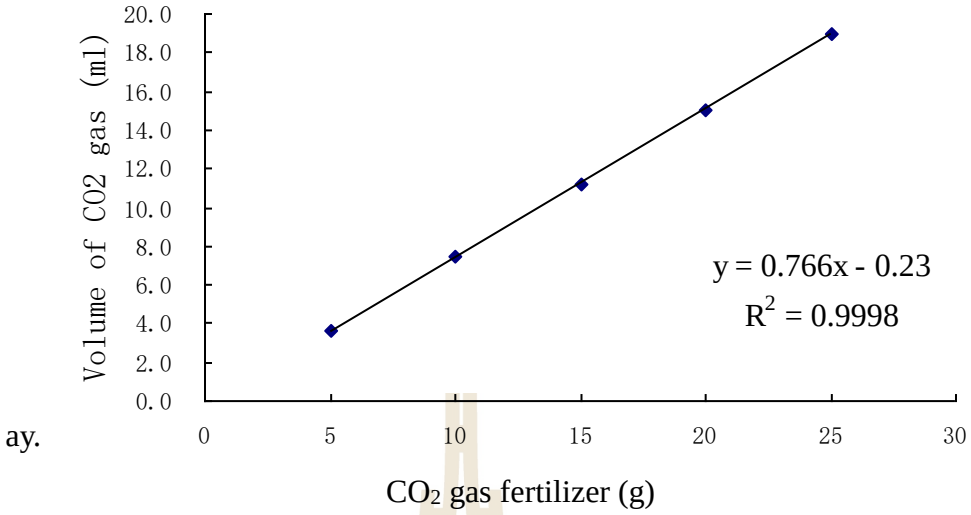


Figure 5.4 The volume of released CO_2 gas by CO_2 gas fertilizer at different



ay.

Figure 5.5 The release of CO_2 gas from CO_2 gas fertilizer within 15 days.

The increase in the concentration of atmospheric CO_2 is likely to have a significant effect on plant photosynthesis, metabolism and development **(**Ainsworth and Long, 2005; Nowak *et al.*, 2004). Because of anatomical and functional differences between C_3 and C_4 species, C_4 leaves have higher concentration of CO_2 in the bundle sheath cells. Some authors assumed that C_4 photosynthesis was saturated at ambient concentration of CO_2, and that C_4 plants would be less affected (or not at all) by increased CO_2 gas than C_3 plants **(**Bowes, 1993; Ehleringer and Pearcy, 1983). On the other hand, Vu *et al* (2006) observed an increase in sugarcane photosynthesis grown under elevated CO_2. They studied the response of single sugarcane leaves under elevated CO_2 and well-watered conditions and found significant effects on gas exchanges, photosynthetic enzyme activities, sucrose metabolism, protein and chlorophyll contents **(**Vu *et al.*, 2006b). Sugarcane grown under elevated CO_2 shown an increase of about 30% in sugarcane photosynthesis and 17% in height, and

accumulated 40% more biomass in comparison with the sugarcane grown at ambient CO_2 **(**De Souza *et al.,* 2008). Furthermore, Guan (2007) reported that the suitable density of CO_2 gas from 2000 to 4000 ul/L was suitable for begonia grown *in vitro*. In sugarcane sugar-free tissue culture, CO_2 concentration in culture room was kept at 1500 ul/L (four times the atmospheric CO_2 concentration) during photoperiod *in vitro* indicated the growth of plantlets was four to seven times than grown at atmospheric CO_2 concentration **(**Xiao *et al.,* 2003). All mentioned above indicates that enhance the concentration of CO_2 would increase the growth and development of sugarcane plantlets *in vitro*.

Thereafter, in order to evaluate the combination of Qianxing No.1 and CO_2 gas fertilizer on the growth and development of sugarcane explants in sugar- free medium. It is necessary to determine suitable dose of CO_2 gas fertilizer in the carbon dioxide gas fertilizer culture bottle with 250ml volume **(**Lu *et al.,* 2013) for next research. Depending on the case of density of CO_2 gas from 2000 to 4000 ul/L was suitable for begonia grown *in vitro* and 1500 ul/L CO_2 concentration in culture room would promote the growth of sugarcane *in vitro* respectively, by the linear regression equation $y=0.766x-0.23$ ($R^2=0.9998$) as Figure 5.5 shows, the suitable dose of CO_2 gas fertilizer could be determined at 0.9g, 1.0g and 1.1g, and the density of CO_2 gas in culture bottle was equal to 1837.6 ul/L, 2144.0 ul/L and 2450.4 ul/L respectively.

5.4.2 Results of RSD and SCMV detection in sugarcane plantlets

Sugarcane is one of the world's major food-producing C_4 crops. It provides about 75% of the sugar harvested for human consumption (Food and Agriculture Organization statistics) 2013. SCMV and RSD are two serious sugarcane diseases all over the world **(**Li *et al.,* 2014; Wang *et al.,* 2009; Xu *et al.,* 2008). Disease incidence

is generally over 30% and can be as high as 100% in susceptible cultivars in the worst infected fields, causing a decrease of 11-35% in sugarcane stems germination rate, culminating in a loss of 3-50% of the total cane yield and a decrease of 6-14% in the sucrose content **(**Huang and Li, 2011; Huang *et al.,* 2007). To produce disease free sugarcane plantlets for farmer, it is necessary to detect the infection of RSD and SCMV before large scale production disease free sugarcane plantlets for cultivating in field.

From Figure 5.6 and Figure 5.7, we can see that sugarcane RSD and SCMV diseases were detected by PCR with the two pairs of specific primers Cxx1-Cxx2 which gave 438bp nucleotides **(**Pan *et al.,* 1998). While the SCMV-F & SCMV-R gave 924bp nucleotides with the SCMV sample **(**Tang, 2006). These results indicated that, although the sugarcane stems were disinfected by heat treatment as section 4.3.1 described, sugarcane RSD and SCMV diseases still persisted in some of the explants at the beginning of culturing process. Its critical employed disease free explants continue the subculture.

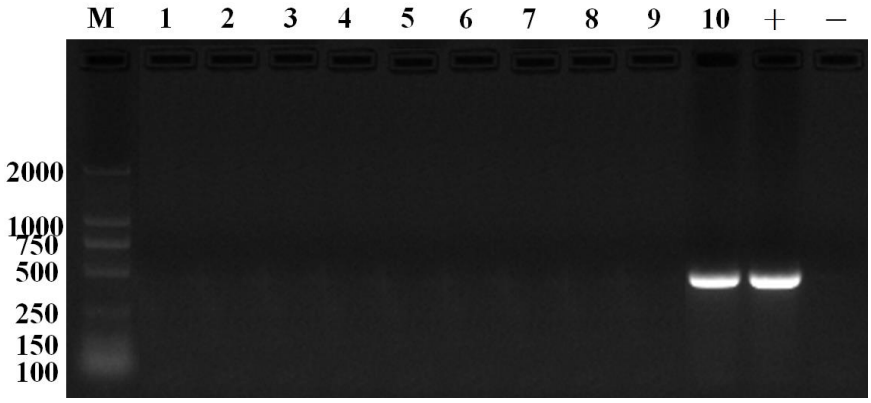


Figure 5.6 PCR assay for detection of RSD bacteria in first generation multiple sugarcane cluster buds which were generated from single original bud. M: Marker; 1-10: Cluster buds samples; +: Positive control; —: Negative control.

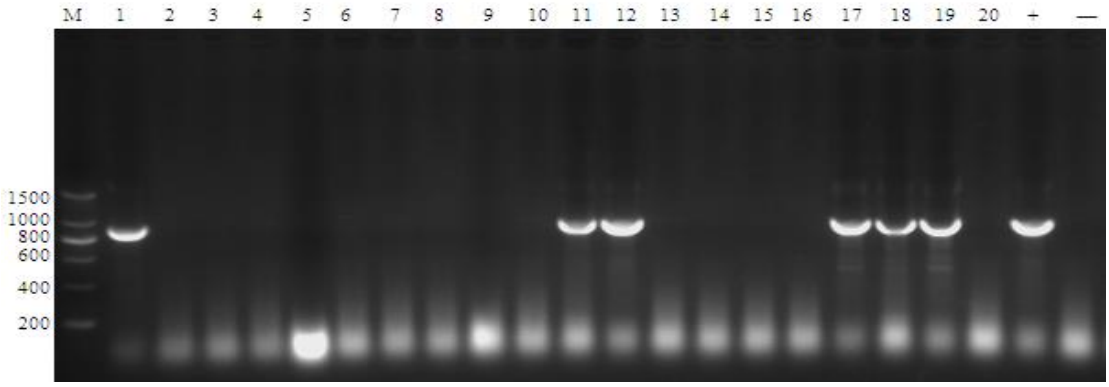


Figure 5.7 RT-PCR assay for detection of SCMV virus in first generation multiple sugarcane cluster buds which were generated from single original bud. M: Marker; 1-20: Cluster buds samples; +: Positive control; —: Negative control.

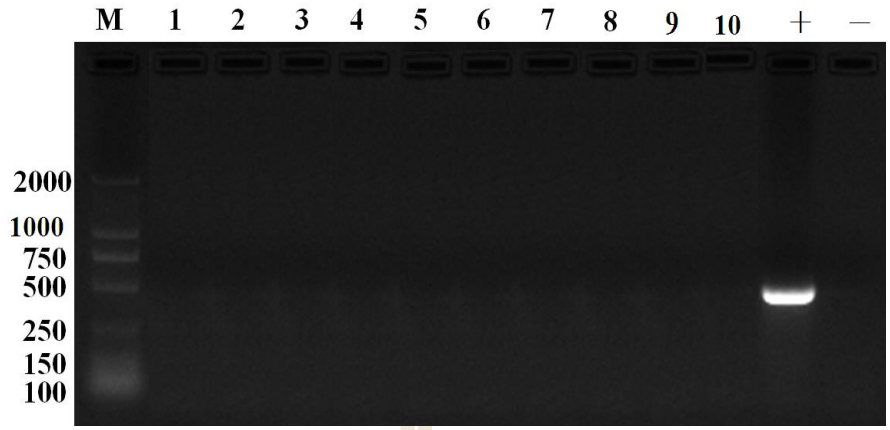


Figure 5.8 PCR assay for detection of RSD bacteria in rooting sugarcane plantlets. M: Marker; 1-10: Cluster buds samples; ＋: Positive control; －: Negative control.

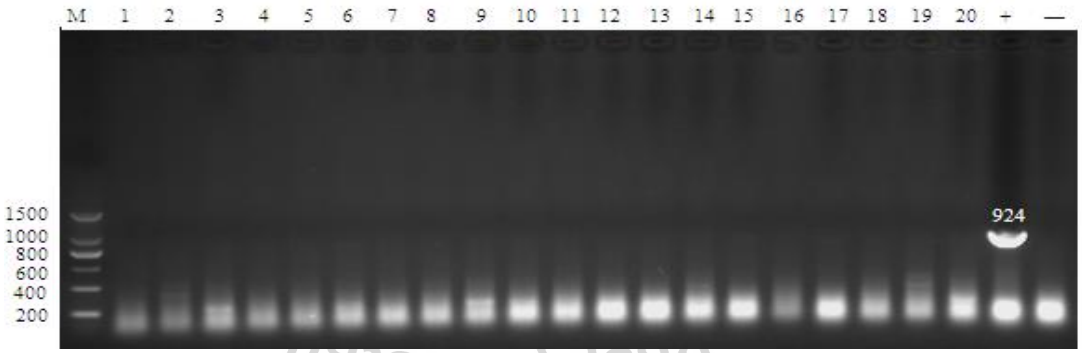


Figure 5.9 RT-PCR assay for detection of SCMV virus in rooting sugarcane plantlets. M: Marker; 1-10: Cluster buds samples; ＋: Positive control; －: Negative control.

Fortunately, as shown in Figures 5.8 and 5.9, in the second multiple culturing process, when chosen sugarcane disease free cluster buds as explants to produce plantlets, no sugarcane RSD and SCMV were detected from rooting plantlets. It was not only demonstrated that the disease detection in the process of disease-free plantlets produced was critical, but also indicated that plant tissue culture technology is a better way to produce disease free plantlets.

5.4.3 Combination of Qianxing No.1 and CO_2 gas fertilizer on the growth and development of sugarcane explants in sugar-free medium

5.4.3.1 Growth and development of explants in multiplication phase

From the previous results of section 4.5 and 5.4.1, the dose levels of CO_2 gas fertilizer would be determinate at C1 (0.9g), C2 (1.0g), C3 (1.1g) and the concentration levels of Qianxing No.1 (200X) would be determinate at B1 (0.4%), B2 (0.5%), B3 (0.6%). CO_2 gas fertilizer and Qianxing No.1 were combined on the growth and development of sugarcane explants in multiplication phase sugar-free media.

It could be seen from Table 5.2 that with the increase of 200X Qianxing No.1 concentration from 0.4% to 0.6%, percentage of contamination in multiplicative non-autoclaved sugar-free medium reduced from 2.67% to 4.0%. This was different from the result reported by Lu *et al* (2012) that contamination rate was 5.67% to 11.0% under the same concentration of Qianxing No.1 and same explants of sugarcane variety *Qiantang* No.5. It might be the addition of sugar in medium in previous researches, but in the present study there was no sugar in the media, and the risk of microbial contamination would be reduced to its minimum. The contamination 7.0% of CK was significantly higher than all the treatments contamination 2.67-4.0%,

it was indicated that the combination of Qianxing No.1 and CO_2 gas fertilizer would overcome contamination in low-cost open tissue technology when compared with conventional one. This view was supported by Kozai and Iwanami (1988) reported low concentration of sugar with low contamination in carnation tissue culture. The survival rates showed no significant differences among all the treatments, but significantly higher than CK, that's because most plantlets with rapid growth and good development *in vitro*, had an ability to overcome the inhibition of microbes as well as inhibition of higher concentration of Qianxing No.1. But the bud numbers and propagation efficiency of C2B2 and CK was significantly higher than other treatments, it indicated that sugarcane plantlets could take benefit from the combination of CO_2 gas fertilizer and Qianxing No.1, and the sugar in the media would be replaced by CO_2 gas fertilizer.

Moreover, under the concentration of B2, average buds number were significantly different among the treatments of C1B2, C2B2 and C3B2, trending to a parabolic variation, which reached the peak 314.7 buds when the dose of CO_2 gas fertilizer was 1.0g per culture bottle. But it declined to 288.7 buds with the dose of CO_2 gas fertilizer increased to 1.1g per culture bottle, and similar results were obtained from the propagation efficiency of sugarcane plantlets. Such a result not only suggested that sugarcane explants had rapid growth and good development under photo-autotrophic culture environment in media without sugar but CO_2 enrichment in the multiplication phase **(**George and Manuel, 2013), but also demonstrated that they were less susceptible to microbial contamination when incorporated with Qianxing No.1 in sugar-free media at the concentration of 0.5%.

Table 5.2 Explants development observed after 20 days in multiplicative phase under the combination of CO_2 gas fertilizer with Qianxing No.1.

Treatments	Contamination (%)		Surviving (%)		Number of bud		Propagation efficiency	
C1B1	4.00	b	95.67	a	271.3	f	2.71	f
C2B1	4.00	b	95.67	a	278.7	e	2.79	e
C3B1	4.33	b	95.33	a	282.0	de	2.82	de
C1B2	3.50	bc	96.33	a	304.3	b	3.04	b
C2B2	3.33	bc	96.33	a	314.7	a	3.15	a
C3B2	3.17	bc	96.67	a	288.7	c	2.89	c
C1B3	2.67	c	95.00	a	272.7	f	2.73	f
C2B3	2.83	c	95.33	a	306.7	b	3.07	b
C3B3	2.83	c	95.00	a	283.0	d	2.83	d
CK	7.0	a	93.00	b	312.0	a	3.12	a
CV (%)	8.47		0.53		0.73		0.73	

(1) Means followed by the small letters in the column do not differ by Duncan's test ($P<0.05$). Bud contaminate and surviving percentage were transformed to arc sin $(x/100)^{½}$ for the analysis of variance.

(2) Levels of CO_2 gas fertilizer were C1 (0.9g), C2 (1.0g), C3 (1.1g). Levels of 200X *Qianxing N0.1* were B1 (0.4%), B2 (0.5%), B3 (0.6%).

5.4.3.2 Growth and development of explants in rooting phase

During the rooting phase, among all the treatments, microbial contamination rate tended to show a declined linear response with the increase of Qianxing No.1 concentrations in sugar-free media as shown in Table 5.3. Moreover, the contamination 7.1% of CK was significantly higher than all the treatments contamination 2.67-4.0%, it was indicated that Qianxing No.1 would be a powerful antimicrobials in low-cost open tissue technology. The results were in agreement with the observation of Lu *et al* (2014) who had reported that microbes infection rates were declined with the increase of Qianxing No.1 concentrations. Although the survival rate of sugarcane plantlets belonging to C2B2 treatment did not have higher significances compared to other treatments except for C1B3 and C2B3, the average number of roots and leaves appeared higher significantly than rest treatments with the increase of CO_2 gas fertilizer from C1 to C3, and they reached the peak of 14.7 roots and 7.9 leaves per plantlet respectively. It was due to the sugarcane plantlets with the ability of photoautotrophic and reached the carbon dioxide saturation point *in vitro* when supplied 1.0g CO_2 gas fertilizer. Furthermore, the number of roots (11.9-14.7) and leaves (5.6-7.9) of all the treatments were significantly higher than CK 10.1 roots and 5.0 leaves respectively, that indicated the combination of CO_2 gas fertilizer and Qianxing No.1 could promoted the growth and development of plantlets in vitro, when compared with conventional tissue culture.

These results were supported by the reports that follow. On the one hand, most chlorophyllous explants/plants *in vitro* had the ability to grow photo autotrophically (without sugar in the culture media), and that the low or negative net photosynthetic rate of plants *in vitro* was not due to poor photosynthetic ability, but to

the low CO_2 concentration in the air-tight culture vessel during the photoperiod **(**Xiao *et al.*, 2011). On the other hand, De Souza *et al* (2008) reported that sugarcane grown under elevated (720 ppm) CO_2 concentration showed an increase of about 30% in photosynthesis and accumulated 40% more biomass in comparison with the concentration grown at ambient (370 ppm) CO_2 concentration **(**De Souza *et al.*, 2008).

Table 5.3 Explants development observed after 25 days in rooting phase under the combination of CO_2 gas fertilizer with Qianxing No.1.

TRT	Contamination (%)	Surviving (%)	Number of Roots	Number of Leaves	Height (cm)
C1B1	4.00 b	95.67 ab	11.9 e	5.6 e	6.6 ab
C2B1	4.00 b	95.67 ab	13.0 bc	5.7 de	6.7 ab
C3B1	4.00 b	95.67 ab	12.5 cd	6.3 bc	6.6 ab
C1B2	3.50 bc	96.33 ab	12.1 de	6.9 b	6.6 ab
C2B2	3.50 bc	96.67 a	14.7 a	7.9 a	6.6 ab
C3B2	3.00 bc	95.67 abc	12.7 bc	6.3 bc	6.8 a
C1B3	2.67 c	93.00 cd	12.4 cd	5.8 cde	6.5 b
C2B3	2.67 c	94.33 bcd	13.1 b	6.3 bcd	6.7 ab
C3B3	2.67 c	94.67 abc	12.6 bcd	6.0 cde	6.7 ab
CK	7.10 a	93.00 d	10.1 f	5.0 f	6.6 ab
CV (%)	10.13	0.56	2.52	5.46	1.96

(1) Means followed by the small letters in the column do not differ by Duncan's test ($P<0.05$). Bud contaminate and surviving percentage were transformed to arc sin $(x/100)^{½}$ for the analysis of variance.

(2) Levels of CO_2 gas fertilizer were C1 (0.9g), C2 (1.0g), C3 (1.1g). Levels of 200X Qianxing N0.1 were B1 (0.4%), B2 (0.5%), B3 (0.6%).

As pervious research demonstrated, open tissue culture and sugar-free tissue culture had many advantages including promotion of growth (photosynthesis), high survival percentage, little loss of plantlets due to microbial contamination, and reduction in labor cost. Meanwhile, the disadvantages of sugar-free tissue culture included relative complexity of techniques and knowledge required for controlling *in vitro* environment as well as the expense for lighting, CO_2 enrichment and cooling **(**Kozai *et al.*, 2005; Xiao *et al.*, 2011). Moreover, in open tissue culture, the sugar which was added in media would offer a chance of survival for microbes *in vitro* **(**George and Manuel, 2013).

From these points of view described above, the present experiment results might suggest that such an efficient plant tissue culture technique would overcome the disadvantages of sugar-free tissue culture and open tissue culture while it could combine their advantages by the application of CO_2 gas fertilizer in sugar-free tissue culture. However, this has to be taken with caution as the most important factor: the present experiment was not performed in inducing phase, but laid out during multiplication phase and rooting phase *in vitro*. In other words, the application of CO_2 gas fertilizer in sugar-free tissue culture required the explants with the ability of photoautotrophic *in vitro*.

5.4.3.3 Measurement net of photosynthesis of sugarcane plantlets

Plant tissue culture has contributed to producing virus-free, genetically superior plantlets. However, its commercial application is still limited due to its relatively high production costs. The high costs are due to low photosynthetic photon flux (PPF), low growth rate *in vitro* by using an airtight culture vessel and loss of plantlets *in vitro* by microbial contamination. All of which contribute to a low net

photosynthetic rate (Pn) of plantlets *in vitro* **(**Heo and Kozai, 1999). Low photo-synthetic ability of plantlets *in vitro* is considered to be the cause of low survival percentage and the slight weight per plantlet *in vitro* **(**Xiao and Kozai, 2006b).

The net photosynthesis of plantlet was measured by the modified IRGA (LI-6400XT) which was able to rapid measurement net photosynthesis of whole plantlet *in vitro* as shown in Figure 5.3. From Table 5.4 and Figure 5.10, it was found that the net photosynthesis and weight per plantlet which was produced by new plan tissue culture method was significant higher than traditional tissue culture. It was due to the synergistic effect of CO_2 gas fertilizer and Qianxing No.1 in the new tissue culture system (named improved open tissue culture system or improved sugar-free tissue culture system). On the one hand, Qianxing No.1 controlled the microbial contamination. On the other hand, CO_2 gas fertilizer enhanced the concentration of CO_2 gas in carbon dioxide gas fertilizer culture bottles **(**Lu *et al.*, 2013) compared with conventional tissue culture. Moreover, the average net photosynthesis of three plantlets per bottle was significant greater than five plantlets per bottle. It was due to wide growth space means well illumination for each leaf and enough nutrition for well development of plantlets when compared with high culture densities *in vitro* condition. This view was demonstrated by the average weight of plantlets with different culture densities as shown in Table 5.4 and Figure 5.11, the average weight of three plantlets per bottle was significant greater than five plantlets per bottle.

Table 5.4 Net photosynthesis and weight of plantlet observed after 25 days in the rooting phase under the combination of CO_2 gas fertilizer with Qianxing No.1.

Plantlets/bottle	Culture type	Pn (µmol/s.plantlet)	Weight/plantlet (g)
Five	Traditional	10.80f	1.37f
	New method	17.44c	2.03c
Four	Traditional	11.22e	1.57e
	New method	19.54b	2.27b
Three	Traditional	13.35d	1.77d
	New method	20.28a	2.53a
CV (%)		0.81	3.0

Plant tissue culture has contributed to producing virus-free, genetically superior plantlets. However, its commercial application is still limited due to its relatively high production costs. The high costs are due to low photosynthetic photon flux (PPF), low growth rate *in vitro* by using an airtight culture vessel and loss of plantlets *in vitro* by microbial contamination. All of which contribute to a low net photosynthetic rate (Pn) of plantlets *in vitro* **(**Heo and Kozai, 1999). Low photosynthetic ability of plantlets *in vitro* is considered to be the cause of low survival percentage and the slight weight per plantlet *in vitro* **(**Xiao and Kozai, 2006b).

The net photosynthesis of plantlet was measured by the modified IRGA (LI-6400XT) which was able to rapid measurement net photosynthesis of whole plantlet *in vitro* as shown in Figure 5.3. From Table 5.4 and Figure 5.10, it was found that the net photosynthesis and weight per plantlet which was produced by new plan tissue culture method was significant higher than traditional tissue culture. It was due

to the synergistic effect of CO_2 gas fertilizer and Qianxing No.1 in the new tissue culture system (named open sugar-free tissue culture system). On the hand, Qianxing No.1 controlled the microbial contamination. On the other hand, CO_2 gas fertilizer enhanced the concentration of CO_2 gas in carbon dioxide gas fertilizer culture bottles **(**Lu *et al.*, 2013) compared with conventional tissue culture. Moreover, the average net photosynthesis of three plantlets per bottle was significant greater than five plantlets per bottle. It was due to wide growth space means well illumination for each leaf and enough nutrition for well development of plantlets when compared with high culture densities *in vitro* condition. This view was demonstrated by the average weight of plantlets with different culture densities as Table 5.4 and Figure 5.11 shows, the average weight of three plantlets per bottle was significant greater than five plantlets per bottle.

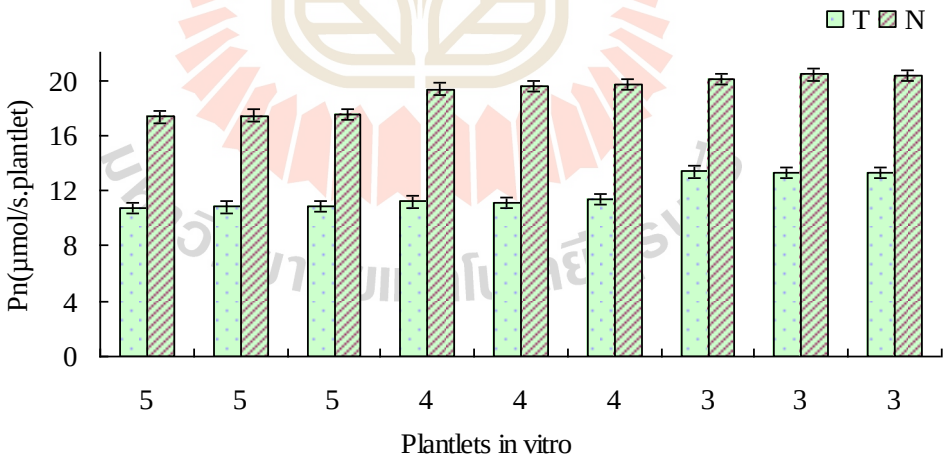


Figure 5.10 Net photosynthesis rates of sugar-free sugarcane plantlets *in vitro*. T: traditional tissue culture; N: Low-cost open tissue culture.

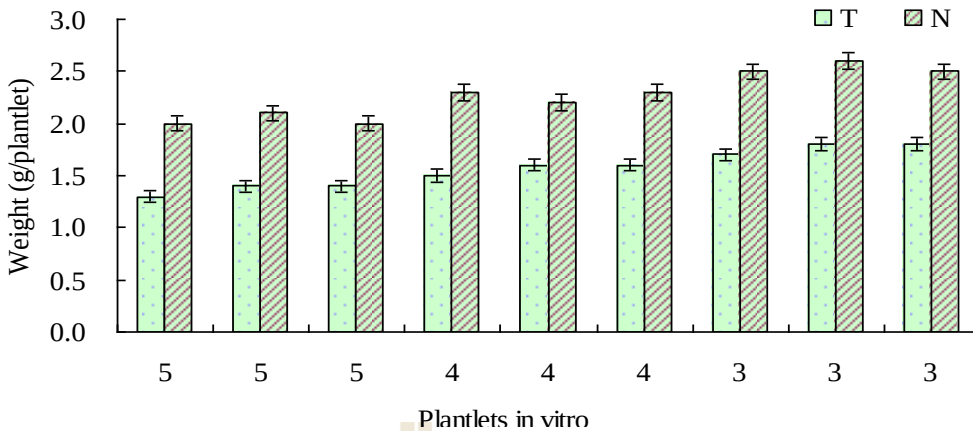


Figure 5.11 Weight of sugar-free sugarcane plantlets *in vitro*. T: Traditional tissue culture; N: Low-cost open tissue culture.

5.4.4 Carbon dioxide gas fertilizer culture bottle

In present studies, our research group has designed the carbon dioxide gas fertilizer culture bottle (Patent No: ZL 2012 2 0277809.5) **(**Lu *et al.*, 2013) which was able to overcome the defects including: natural ventilation through the gaps of the contact surfaces of the vessel is low effective in gas enrichment and might be the increase of microbial contamination. Besides, in forced ventilation system, the growth in the vessel was not uniform, with larger plants near the air inlet and comparatively smaller plants near the air outlet **(**Heo and Kozai, 1999). Moreover, it is critical that both of them required sterilized culture media by autoclave and does not lower the cost of plantlets.

It was due to the carbon dioxide gas fertilizer culture bottle taken benefits from CO_2 gas fertilizer and Qianxing No.1. The results of Table 5.3 and Table 5.4 shown, on the one hand, CO_2 gas fertilizer is able to slow release CO_2 gas under the stimulation of light and then enhanced the CO_2 concentration *in vitro*. On the other

hand, Qianxing No.1 was able to efficient control the microbial contamination at 3.5% and friendly with plantlets in media. Our result was supported by Cui (2005) who was reported that produced grape, apple, potato and butterfly orchid plantlets *in vitro* by open tissue culture with the low contamination less than 10%.

5.4.5 Method and device of rapid determination net photosynthetic of plantlet *in vitro*

Green plants can transform sunlight energy to chemical energy by means of photosynthesis. During the process, plants fix carbon dioxide (CO_2) and release oxygen (O_2) while coping with the loss of water (H_2O). Measurements of photosynthesis are needed for comparing and under-standing productivity (biomass accumulation) of vegetal systems at the leaf, plant or community level as well as their response to environmental stresses. The common methods of photosynthesis measure including: originally estimated based on the accumulation of dry matter from the point of germination to the time it is cut in order to make the measurement as mentioned by Hodson *et al* **(**2005); electrochemical sensor method；gas exchange method; carbon dioxide isotopes method; photosynthesis estimation by modeling; acoustic waveguide method; fluorescence based techniques and phytomonitoring application **(**Millan-Almaraz *et al.*, 2009).

It is important to study the photosynthetic rate of whole plant tissue culture in controlled environment conditions for improving the culture environment, adjusting the culture medium, enhancing industrial production of tissue culture for agricultural production by improve plantlets survival after transplanting in field. In recently, photosynthesis measuring device mostly measured a small part of the leaf area (approximately 6.0 cm^2) photosynthetic rate. However, most of experiments required me-

asuring the photosynthetic rate of whole plantlet *in vitro* at different times, different light intensity and different CO_2 gas concentration conditions. Consequently, it is necessary to find a fast and accurate method to measure the photosynthetic rate of whole plantlets.

Follow the principle of gas exchanges, our research group modified the photosynthesis measure system base on IRGA (LI-6400XT). As the results of Table 5.4 and Figure 5.10 shown, the present invention of method and device were not only able to rapid determinate the real time net photosynthetic of whole plantlet *in vitro*, but also keep the measured plantlets without any mechanical compression injury during measures. Moreover, such the method and device of rapid determination net photosynthetic was able to measure the respiration rate of fruits, like mango, apple and banana and so on.

5.5 Conclusion

The rule of CO_2 gas fertilizer releases CO_2 gas would be determinated by a pair of cylinders in the culture environment. The growth and development of sugarcane plantlets *in vitro* indicated that the synergistic effect between CO_2 gas fertilizer and Qianxing No.1 were displayed very well during explants multiple and rooting phase in open sugar-free culture by employed carbon dioxide gas fertilizer culture bottle as culture flask. Meanwhile, the present results demonstrated that CO_2 gas fertilizer would be a new source of CO_2 gas to increase the concentration of CO_2 and enhance the net photosynthesis of plantlets in flask. Besides, the invention of method and device were not only able to rapid determinate the real time net photosynthetic of whole plantlet *in vitro*, but also keep the measured plantlets without

any mechanical compression injury during measures. Moreover, for produce disease- free plantlets, during the early stage of tissue culture process, it was critical to detect the infection of sugarcane RSD and SCMV diseases by the specificity primers Cxx1, Cxx2 and SCMV-F, SCMV-R respectively.

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CHAPTER VI

STUDY THE EFFICIENCY OF LOW-COST OPEN TISSUE CULTURE TECHNOLOGY IN SUGARCANE PROPAGATION

6.1 Abstract

The objective of this section was to evaluate the efficiency of open sugar-free tissue culture technology in sugarcane propagation by combining Qianxing No.1 and 1.0g CO_2 gas fertilizer in carbon dioxide gas fertilizer culture bottle when comparing with conventional plant tissue culture. In the present of open sugar-free tissue culture, 200X Qianxing No.1 with the concentration of 0.5% was combined with 1.0 g CO_2 gas fertilizer (equal 2144.0 μl/L CO_2 gas in 250ml carbon dioxide gas fertilizer culture bottle) on sugarcane tissue culture. As a result, the plantlets produced by open sugar-free tissue culture were stronger than those by conventional tissue culture, and the cost of production was considerably reduced to RMB 0.40 per plantlet when compared with conventional plant tissue culture for in the production scale of 10,000 sugarcane plantlets. Conclusion, compared with conventional tissue culture, open sugar-free tissue culture system was built up with the advantages of low contamination, simple operation steps, high quality and low cost of sugarcane plantlets, etc.

6.2 Introduction

Plant tissue culture is primarily based on rapid multiplication of tiny stem cuttings, axillary buds, somatic embryos, cell clumps in suspension cultures and bioreactors. The plants can be multiplied anywhere under controlled environmental conditions throughout the year irrespective of the season and weather **(**Etienne *et al.*, 1999). The cultured cells and tissue can take several pathways. The pathways that lead to the production of true-to-type plants in large numbers are the preferred ones for commercial multiplication.

However, tissue culture technology is more expensive than the conventional method of plant propagation because of the initial capital input followed by maintenance of the high-tech equipment involved and the controlled conditions required in addition to the skilled labor **(**Ahloowalia and Savangikar, 2004). The main bottleneck is the high cost of tissue culture planting materials compared to the conventionally propagated saplings. It is a capital-intensive industry, and in some cases the unit cost per plant becomes unaffordable **(**George and Manuel, 2013).

Hence, it is necessary to adopt strategies to reduce production cost and lower the cost per plantlet. In this section, the efficiency of improved open tissue culture technology in sugarcane propagation was evaluated by comparing with conventional plant tissue culture.

6.3 Materials and Methods

6.3.1 Materials

The axillary buds of sugarcane variety of *Qiantang 5* were explants. The carbon dioxide gas fertilizer culture bottles and Qianxing No.1 were come from

chapter V and Chapter III respectively. CO_2 gas fertilizer was provided by Beijing Jin Ling Shuang Environmental Protection Equipment Company Limited., China.

6.3.2 Methods

The different efficiency between low-cost open tissue culture and conventional ones in sugarcane propagation were compared based on the total amount of 10,000 sugarcane planets for each tissue culture techniques.

6.3.3 Data collection and analysis

Base on the total of 10,000 sugarcane plantlets, the cost was observed. On the one hand, such as depreciation of fixed assets, including autoclave, clean bench, culture bottle, air-conditions, culture shelves, isolate room and culture room and so on, where the depreciation rate of equipments was 10% per annum **(**Cui, 2005; Tiwari *et al.*, 2012). On the other hand, direct cost of production, such as charge of electric for media sterilize, explants inoculate, Qianxing No.1, CO_2 gas fertilizer, MS media, hormones, water as well as labor cost, etc.

6.4 Results and Discussion

The cost of plantlets by using traditional tissue culture and open sugar-free tissue culture was calculated. Table 6.1 shows the recurring expenses incurred on various items for producing a batch of 10,000 sugarcane plantlets. It is apparent from the table that a total amount of RMB(￥) 10,000.00 and RMB(￥) 6,000.00 is required against recurring expenses for each lot of 10,000 sugarcane plants if medium sterilization by autoclaving and Qianxing No.1 respectively were followed.

Qianxing No.1 sterilization of media saved RMB(￥) 4,000.00 for each lot of 10,000 sugarcane plantlets, which includes electricity cost on autoclaving and

isolating, depreciation value of autoclave, clean bench, media (because of Qianxing No.1 could reduce the microbial contamination, then saving media) and savings on manpower by enhancing working efficiency in a relative aseptic lab compared with in clean bench. Furthermore, Figure 6.1 shows that the plantlets which were produced by open sugar-free tissue culture with more roots and leaves are stronger than those by conventional tissue culture. It means that the well developed plantlets would be high survival rate and well acclimatization after transfer to field when comparing with conventional tissue culture **(**Ahloowalia and Savangikar, 2004).

Thus, the cost of production is considerably reduced by RMB(￥) 0.40 per plantlet, in other words, saving 40% cost of each lot of 10,000 plantlets. In this way a total saving of RMB(￥) 0.40 million can be made annually in a laboratory with a production capacity of 1.0 million plantlets per year following the open sugar-free tissue culture instead of conventional tissue culture.

Our result was different from these reports that in the presence of NaClO at 0.1% concentration in non-autoclaved media for sugarcane micropropagation where saved 19.62% cost of each lot of 10,000 sugarcane plantlets, where the depreciation cost of equipments were 10% per annum **(**Cui, 2005; Tiwari *et al.*, 2012). It might be two main items, Qianxing No.1 which were made of chemical suppressants and botanical fungicide are friendly with plantlets, better than single chemical suppressants like sodium hypochlorite on controlling microbial contamination **(**Cui, 2005). On the other hand, CO_2 gas fertilizer promoted the growth and development of sugarcane plantlets by increasing the net photosynthesis in CO_2 gas culture bottle.

The results suggested that Qianxing No.1 sterilization of media and application of CO_2 gas fertilizer may be adopted as a cost effective option of autoclave

sterilization during sugarcane micropropagation at commercial scale. It would not only save expenses on autoclaves and electricity bills, but also save time and labor spent on autoclaving and isolating. Consequently, the technique would be more efficient and cost effective for sugarcane tissue culture on commercial scale.

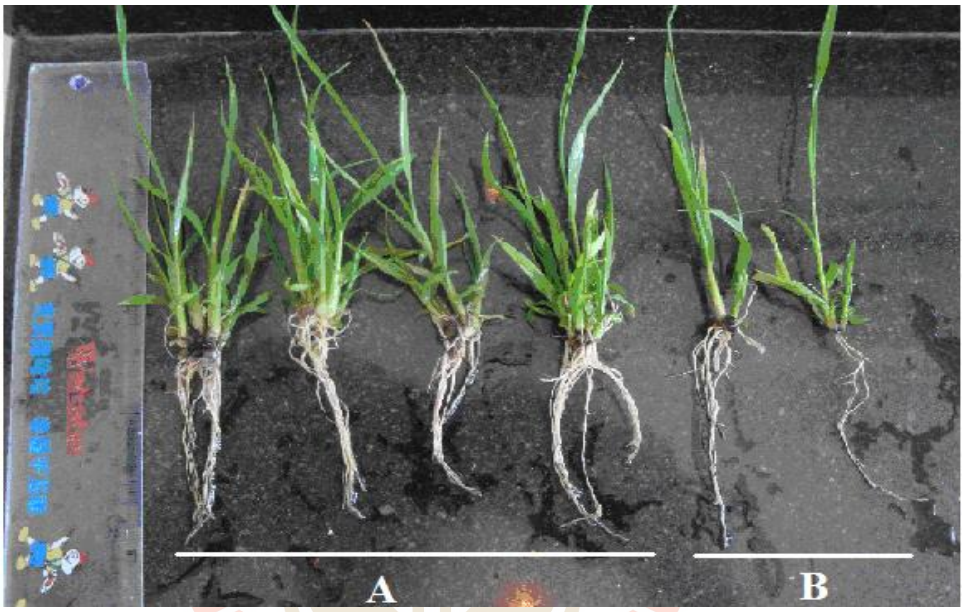

Figure 6.1 The plantlets by (A) low-cost open tissue culture and (B) conventional tissue culture technique produced.

Table 6.1 The cost of production of plantlets by two tissue culture techniques.

Subject	Traditional tissue culture	Low-cost open tissue culture
1) Depreciation of fixed assets	(RBM￥)	
Autoclave	150.00	---
Clean bench	150.00	---
Other	200.00	200.00
2) Cost of production		
Charge of media sterilize	900.00	---
Charge of electric for explants inoculate	200.00	---
Antimicrobial compounds	---	350.00
CO_2 gas fertilizer	---	300.00
Media (MS + hormones)	5000.00	3800.00
Culture bottle	100.00	150.00
Labor cost	3000.00	900 .00
Other	300.00	300.00
3) Total	10000.00	6000.00

Note: CO_2 gas culture bottles are expensive than normal culture bottles. Based on recurring expenses incurred on various items for producing a batch of 10.000 sugarcane plantlets (1.0￥= 5.3071 ฿ on October 29, 2014).

6.5 Conclusion

By the depreciation rate of equipments 10% per annum, open sugar-free tissue culture was able to save 40% cost per plantlet (including saving charge of media sterilize, charge of electricity for explants inoculate, basal MS media and hormones, labor cost) when compared with conventional tissue culture for each lot of 10,000 sugarcane plantlets. The open sugar-free tissue culture has been successfully utilized for production of sugarcane plantlets on large scale. This has helped in reducing the cost of plantlets to a considerable extent.

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CHAPTER VII

CONCLUSION

In this study, the advantages of open tissue culture and sugar-free tissue culture were combined by CO_2 gas fertilizer in carbon dioxide gas culture bottle, and the effect of this combination on sugarcane tissue culture was evaluated when compared with conventional tissue culture. Results as following:

1. Five common fungus species (*Mucor* sp, *Alternaria* sp, *Penicillium* sp, *Aspergillus flavus* and *Rhizopus* sp) and one bacterium species *Bacillus* sp were isolated from laboratory environment and employed as test microbes in this research.

2. A broad-spectrum antimicrobial compounds named Qianxing No.1 was successfully developed with the concentration of nicotine 40 mg/L, garlic extraction (57.1%) 80 µl/L, carbendazim (80% WP) 60 mg/L and Yi Peilong 14.5 mg/L (equal active chlorine 0.00145%).

3. One gram CO_2 gas fertilizer was able to release 536 µl CO_2 gas under the culture environment within 15 days in carbon dioxide gas fertilizer culture bottle which was designed by our research group.

4. Compared with conventional tissue culture technique, the broad-spectrum Qianxing No.1 was able to establish a low cost tissue culture system through adding 0.5% of Qianxing No.1 (200X) into the open tissue culture sugar media during the shooting and proliferation phase for sugarcane variety *Qiantang 5*.

5. The growth and development of sugarcane plantlets *in vitro* indicated that the synergistic effect of CO_2 gas fertilizer and Qianxing No.1 were displayed very well during explants multiple and rooting phase in sugar-free media by employed carbon dioxide gas fertilizer culture bottle as culture flask.

6. CO_2 gas fertilizer would be a new source of CO_2 gas to increase the concentration of CO_2 and enhance the net photosynthesis of plantlets *in vitro*.

7. The invented method and device were not only able to rapid determinate the real time net photosynthetic of whole plantlet *in vitro*, but also keep the measured plantlets without any mechanical compression injury during measures.

8. For produce disease-free plantlets, during the early stage of tissue culture process, it was critical to detect the infection of sugarcane RSD and SCMV diseases by the specificity primers Cxx1, Cxx2 and SCMV-F, SCMV-R respectively.

9. The plantlets with more roots and leaves which were produced by open sugar-free tissue culture are stronger than those by conventional tissue culture and the cost of production was considerably saved 40%, reduced by RMB 0.40 per plantlet when compared with conventional plant tissue culture for each lot of 10,000 sugarcane plantlets.

APPENDICES

Table 3.4 Ingredients of basal MS medium

Ingredients	Chemical formula	Concentration
Ammonium nitrate	NH_4NO_3	1,650 mg/L
Calcium chloride	$CaCl_2 \cdot 2H_2O$	440 mg/L
Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	370 mg/L
Potassium phosphate	KH_2PO_4	170 mg/L
Potassium nitrate	KNO_3	1,900 mg/L
Boric acid	H_3BO_3	6.2 mg/L
Cobalt chloride	$CoCl_2 \cdot 6H_2O$	0.025 mg/L
Cupric sulphate	$CuSO_4 \cdot 5H_2O$	0.025 mg/L
Ferrous sulphate	$FeSO_4 \cdot 7H_2O$	27.8 mg/L
Manganese sulphate	$MnSO_4 \cdot 4H_2O$	22.3 mg/L
Potassium iodide	KI	0.83 mg/L
Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	0.25 mg/L
Zinc sulphate	$ZnSO_4 \cdot 7H_2O$	8.6 mg/L
Ethylene Diamine Tetraacetic Acid	$Na_2EDTA \cdot 2H_2O$	37.2 mg/L
I-Inositol		100 mg/L
Niacin		0.5 mg/L
Pyridoxine. HCl		0.5 mg/L
Thiamine · HCl		0.1 mg/L
Glycine		2.0 mg/L
Edamin (lactalbumin hydrolysate)		1.0 g/L

Note: Agar 8 g/L, sugar 30g/L, an optimum pH of 5.8 should be maintained.

Table 5.5 Quality Comparation of plantlets in two culture methods

Culture phase	Items	Low-cost open tissue culture	Conventional tissue culture
Inducing	Contamination (%)	6.67	56.67
	Browning (%)	53.33	43.33
	Surviving (%)	80	43.33
Proliferation	Contamination (%)	3.33	7
	Propagation efficiency	3.15	3.12
Rooting	Contamination (%)	3.50	7.10
	Surviving (%)	96.67	92.90
	Roots per plantlets	14.7	10.1
	Leaves per plantlets	7.9	5.0
	Height (cm)	6.6	6.6
	Pn (µmol/s.plantlet)	17.44	10.80
	Weight (g) per plantlets	2.03	1.37

证书号第2614475号

实用新型专利证书

实用新型名称：二氧化碳气肥培养瓶

发　明　人：卢加举;张正学;蒙秋伊;黄先群;张志勇;王健;刘鹏飞
查兰松;雷石富;李向勇;彭仕荣

专　利　号：ZL 2012 2 0277809.5

专利申请日：2012年06月13日

专 利 权 人：贵州省亚热带作物研究所

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局长

2013年01月02日

第1页（共1页）

Figure 6.2 Carbon dioxide gas culture bottle and the certificate of Chinese patent: ZL 2012 2 0277809.5.

证书号第1263210号

发明专利证书

发明名称：植物组培方法及装置

发　明　人：卢加举;张正学;蒙秋伊;黄先群;张志勇;王健;刘鹏飞
查兰松;雷石富;李向勇;彭仕荣

专　利　号：ZL 2012 1 0194132.3

专利申请日：2012年06月13日

专 利 权 人：贵州省亚热带作物研究所

授权公告日：2013年08月28日

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2013年08月28日

第1页（共1页）

Figure 6.3 Open sugar-free tissue culture and the certificate of Chinese patent: ZL 2012 1 01941323.

Figure 6.4 Two inoculation methods and different culture phases. A: Conventional tissue culture, inoculation in clean bench. B: Open sugar-free tissue culture, inoculation in a relative aseptic lab. C: Culture room. D: Multiplication phase. E and F: Rooting phase. G: Plantlets by open sugar-free tissue culture produce. H: Plantlets by conventional tissue culture produce.

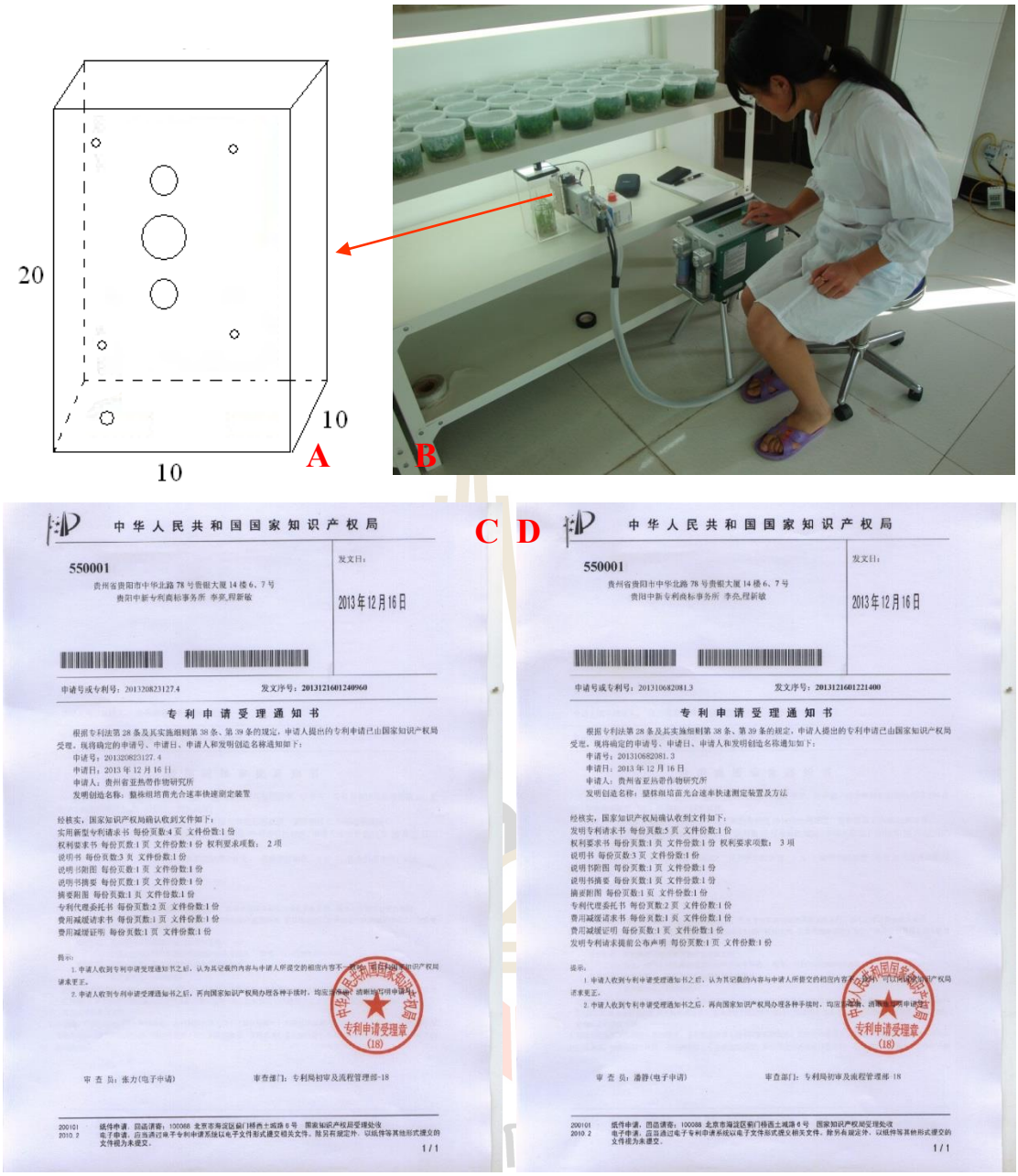

Figure 6.5 Net photosynthetic rapidly determinate system for a whole plantlet *in vitro* and the two accepted notification of Chinese patents. A: homemade leaf chamber. B: Net photosynthetic rapidly determinate system. C: accepted notification of Chinese utility models patent. D: accepted notification of Chinese invention patent.

BIOGRAPHY

Mr. Lu Jiaju was born on June 03, 1982 in Xingyi city, Guizhou province, P.R. China. He received his Bachelor's degree in Horticulture from South China University of Tropical Agriculture in 2004, and Master's degree in Crop Genetics and Breeding from South China University of Tropical Agriculture in 2007. He started his career in Guizhou Institute of Subtropical Crops in 2007; his research field is sugarcane breeding and application. In 2014, he was accepted to the Ph.D. program under the supervision of Asst. Prof. Dr.Arak Tira-Umphon at the School of Crop Production Technology, Institute of Agriculture Technology, Suranaree University of Technology, Thailand.