UNIVERSITI PUTRA MALAYSIA

ESTABLISHMENT OF TISSUE CULTURE AND TRANSFORMATION PROTOCOL FOR TROPICAL CORN (Zea mays L.) INBRED LINES

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ESTABLISHMENT OF TISSUE CULTURE AND TRANSFORMATION PROTOCOL FOR TROPICAL CORN (*Zea mays* L.) INBRED LINES

By

NASIRUDDEEN UMAR MATAZU

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of philosophy

March 2015

DEDICATIONS

To My Late Mum, Hafsah Binti Abu Bakar
And to all those innocent people who died in the hands of terrorist

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

ESTABLISHMENT OF TISSUE CULTURE AND TRANSFORMATION PROTOCOL FOR TROPICAL CORN (*Zea mays* L.) INBRED LINES

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Chairman: Professor Maziah Mahmood, PhD

Faculty: Institute of Tropical Agriculture

Traditional crop improvement alone is insufficient to meet the growing demand of maize hence the need for genetic transformation. Genetic transformation of maize is dependent on the formation of reliable and efficient plantlet regeneration system. However, previously established protocols for tissue culture and genetic transformation are based on maize model genotypes. Hence there is a need to develop a new protocols for these new lines since it's a known fact *in vitro* cultures is majorly dependent genotype. The major objectives of this study was to establish a reliable tissue culture and transformation protocols for some selected tropical CIMMYT maize lines (CML 406, CML 419, CML 425 and CML 427).

Studies performed to evaluate the competence of these lines in responding to *in vitro* stimulus revealed callus initiation, proliferation and maintenance was accomplished in N6 media augmented with 9 μM 2,4-D and 200 mg/L casein hydrolysate, or 5 μM dicamba and 200 mg/L casein hydrolysate in all the genotypes. CML 427 has the highest response with 91.33% followed by CML 419 with 86.67%. The lowest callus induction and proliferation frequency was recorded in CML 425 with 41.67% using 17 days after pollination (DAP) immature zygotic embryo (IZE). Addition of 0.015 mg/L $AgNO_3$, 0.02 mg/L Ag_2SO_4 and 4.12 g/L γ-aminobutyric acid to culture media have significantly increased the formation of embryogenic callus. Studies of callus growth pattern showed all but CML 419 genotype attained maximum growth at 4th week while CML 419 have maximum growth at 3rd week of

culture. Complex organic compounds which included yeast extract, potato extract, coconut water and casein hydrolysate have significantly enhanced callus growth when both fresh and dry weights were measured. Indirect regeneration via organogenesis was accomplished in CML 419 and CML 427 in MS media containing 1 mg/L kinetin, 2 mg/L BAP and 2 mg/L TDZ in separate experimental units. Regeneration efficiencies in these media were found to be 12% and 16.4%; 7.8% and 6.2%; and 3.5% and 4.6% for CML 419 and CML 427 respectively. Both somatic embryogenesis and organogenesis are genotype dependent.

Callus transformation was achieved using *Agrobacterium tumefaciens* strains LBA 4404 harboring pCAMBAIA1304 vector containing (mgfp5-gusA-His6 fusion). Fluorescence microscopy, GUS staining, and PCR amplification of *gfp, gus and hpt II* genes indicated that both GFP and GUS were expressed in the callus. Transformation efficiency was found to be 1.33% and 1.67% for CML 419 and CML 427 respectively. Antioxidative enzyme assays suggest that glutamate dehydrogenase, glutathione reductase, lipid peroxidation and superoxide dismutase activities are significantly ($p \leq 0.05$) higher in test samples than the control. On the other hand, there is no statistically significant difference in catalase and ascorbate peroxidase activities between the test groups and the control.

NMR-based metabolomic studies revealed metabolic differences at different stages of development. The unique compounds discovered in both embryogenic samples are , inositol, choline, proline, β-glucose, asparagine, acetoacetate, ascorbate, aspartate, and phenylalanine. The common metabolites in both embryogenic and organogenic samples from both lines are β-glucose, sucrose, asparagine, aspartate and choline. The major importance of these findings is that organogenic and embryogenic competence in maize is attributed to genotype and cells undergoing embryogenesis and organogenesis evolved special organelles that whose functions remain obscure. Secondly, metabolic data generated could be very useful in understanding these vital processes. These have impressive implications in the basic research especially in cellular and developmental biology. Moreover, this information could be used to identify potential biomarkers, identification gene functions and in drug discovery among others.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEWUJUDAN KULTUR TISU DAN PROTOKOL TRANSFORMASI UNTUK JAGUN TROPIKA (textit Zea mays L.) GARISAN INBRED

Oleh

NASIRUDDEEN UMAR MATAZU

Mac 2015

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Penggunaan transformasi genetik terhadap jagung amat diperlukan untuk penambahbaikan bagi tanaman secara tradisional sahaja masih tidak mencukupi bagi memenuhi permintaan jagung yang semakin meningkat. Transformasi genetik jagung bergantung kepada pembentukan yang betul and efisien bagi sistem penjanaan semula anak pokok. Walaubagaimanapun, protokol yang digunakan sebelum ini dalam kultur tisu dan transformasi genetik kebanyakannya berdasarkan model genotip jagung tersebut. Oleh itu, ia menjadi keperluan untuk membangunkan satu protokol yang baru untuk garisan genotip ini kerana dipercayai kultur tisu hanya bergantung kepada genotip. Objektif utama dalam kajian ini adalah untuk mewujudkan satu protokol yang betul dan transformasi protokol untuk beberapa jagung jenis CIMMYT (CML 406, 419 CML, CML 425 dan 427 CML).

Kajian yang dilakukan untuk menilai keberkesanan jenis jagung tersebut terhadap rangsangan secara in vitro bagi permulaan pembentukan kalus, perkembangan dan penyelenggaraan telah dicapai dengan menggunakan media N6 yang ditambah dengan 9 μM 2,4-D dan 200 mg / L kasein hidrolisat, atau 5 M dicamba dan 200 mg / L kasein hidrolisat dalam semua genotip. CML 427 mempunyai tindak balas yang tertinggi dengan 91.33% diikuti oleh CML 419 dengan 86.67%. Tindak balas bagi permulaan pembentukan dan kekerapan perkembangan kalus adalah 41.67 dengan menggunakan embrio zigot yang tidak matang (IZE) selepas 17 hari pendebungaan (DAP). Penambahan sebanyak 0.015 mg/L AgNO3, 0.02 mg/L Ag2SO4 dan 4.12 g / L γ-aminobutyric asid terhadap media kultur

akan meningkatkan pembentukan kalus embriogenik secara signifikan. Kajian yang dijalankan menunjukkan kesemua kalus menunjukan corak pertumbuhan tetapi genotip CML 419 mencapai pertumbuhan maksimum pada minggu keempat manakala CML 419 pada minggu ketiga. Sebatian kompleks organik termasuk ekstrak yis, ekstrak ubi kentang, air kelapa dan kasein hidrolisat mempunyai perbezaan yang signifikan terhadap pertumbuhan kalus terhadap berat basah dan kering. Pertembuhan secara tidak langsung melaui irganogenesis telah dicapai dalam CML 419 dan CML 427 menggunakan media MS yang mengandungi 1 mg/L Kinetin, 10 μM BAP dan 10 μM TDZ dalam eksperimen yang berasingan. Pertumbuhan secara efisien dalam media didapati 12% dan 16.4%; 7.8% dan 6.2%; dan 3.5% dan 4.6% masing-masing untuk CML 419 dan 427 CML. Kedua-dua embriogenesis somatik dan organogenesis adalah bergantung terhadap genotip.

Tranformasi kalus telah dicapai dengan menggunakan Agrobacterium tumefaciens strain LBA 4404 dengan vektor pCAMBAIA1304 yang mengandungi (mgfp5-gusA-His6 fusion). Mikroskop berpendafluor, GUS penwarnaan, dan amplifi PCR untuk gfp dan gen hpt II menunjukkan bahawa kedua-dua GFP dan GUS telah berkembangan di dalam kalus. Didapati, keberkesanan transformasi adalah 1.33% dan 1.67% bagi CML 419 dan 427 masing-masing CML. Penggunaan esei enzim antioksida seperti glutamat dehidrogenase, glutation reductase, peroksidaan lipid dan superoxide dismutase adalah signifikan ($p \leq 0.05$) berbanding dengan kawalan rawatan. Sebaliknya, tiada signifikan bagi penggunana katalase dan askorbat perixidase antara kumpulan rawatan yang diuji dan kawalan.

Berasakan kajian NMR secara metabolik mendedahkan bahawa perbezaan pada setiap peringkat perkembanga. Sebatian unik yang ditemui pada kedua-dua sampel embriogenik adalah, inositol, choline, proline, -glukosa, asparagine, acetoacetate, askorbat, Aspartate, dan phenylalanine. Metabolit yang sama dalam kedua-dua sampel embriogenik dan organogenic dari kedua-dua garisan adalah -glukosa, sukrosa, asparagine, Aspartate dan choline. Kepentingan utama dalam penemuan ini menunjukkan kecekapan organogenic dan embriogenik dalam jagung adalah disebabkan oleh genotip dan sel-sel yang menjalani embriogenesis dan organogenesis yang telah berkembang menjadi organel khas yang yang fungsinya belum diketahui. Kedua, data metabolik yang dihasilkan boleh menjadi sangat berguna dalam memahami proses kehidupan jagung yang penting. Ini mempunyai implikasi yang mengagumkan dalam penyelidikan asas terutamanya dalam biologi sel dan pembangunan. Selain itu, maklumat ini boleh digunakan untuk mengenal pasti penanda biologi yang berpotensi, mengenalpasti fungsi gen dan penemuan ubat untuk kajian seterusnya.

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I certify that a Thesis Examination Committee has met on 9^{th} March, 2015 to conduct the final examination of Nasiruddeen Umar Matazu on his thesis entitled "Establisment of Tissue Culture and Transformation Protocol for Tropical Corn (*Zea mays* L) Inbred Lines" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the **Degree of Doctor of Philosophy**.

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2iP	6-($\gamma, \gamma-$Dimethylamino)Purine
2,4-D	2,4-Dichlorophenoxyacetic Acid
2,4,5-T	2,4,5-trichloro-phenoxy Acetic Acid
4-CPA	4-chlorophenoxy Acetic Acid
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APX	Ascorbate Peroxidase
AS	Acetosyringone
BAP	6-Benzyloaminopurine
CaMV	Cauliflower Mosaic Virus
CAT	Catalase
CCM	Co-cultivation Media
CE-MS	Capillary Electrophoresis Mass Spectrometry
CIMMYT	*Centrino Internscional Mejoramiento de Maiz Y Trigo*
CML(s)	CIMMYT Maize Line(s)
CPSS	Conditional-Positive Selection Systems
D_2O	Deuterium Oxide
DAP	Days after Pollination
DICAMBA	3,6- Dichloro-2-Methoxy- Benzoic Acid
DMRT	Duncan Multiple Range Test
DIMBOA	2,4- Dihydroxy-7-Methoxy-1,4-Benzoxazin-3-One
DNA	Deoxyribonucleic Acid

DTNB	5,5′-dithio-bis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
DW	Dry Weight
EDTA	Ethylenediaminetetraacetic acid
EPSP	5-Enolpyruvylshikimate-3- Phosphate
EUs	Embryogenic Units
FAA	Formalin-Acetic Acid-Alcohol
FW	Fresh Weights
GABA	γ-aminobutyric Acid
GDH	Glutamate Dehydrogenase
GC-MS	Gas Chromatography Mass Spectrometry
GOI	Gene of Interest
GUS	β-glucuronidase
GR	Glutathione Reductase
IAA	Indole-3- Acetic Acid
IBA	Indole-3- Butyric Acid
IZE	Immature Zygotic Embryo
Kinetin	N-(2-Furanylmethyl)-1H-Purine-6-Amine
LB	Left Border
LC-MS	Liquid Chromatography Mass Spectrometry
LS	Linsmaier and Skoog
MASISH	Maize Seed In Situ Hybridization
MDA	Malondialdehyde

mg/L	Milligram per Liter
MES	2-(4-morpholino)-ethane sulfonic acid
MS	Murashige and Skoog
MVDA	Multivariate Data Analysis
NAA	naphthalene Acetic Acid
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NBT	Nitroblue Tetrazolium
NCPSS	Non Conditional-Positive Selection Systems
NMR	Nuclear Magnetic Resonance
NPII	Neomycin Phosphotransferase II
p-CPA	p-chlorophenoxy Acetic Acid
PEG	Oolyethylene Glycol
PEMs	Proembryogenic Masses
PGRs	Plant Growth Regulators
PICLORAM	4- Amino-3,5,6-Trichloro-Picolinic Acid
PVPP	Polyvinyl Polypyrrolidone
QTL	Quantitative Trait Loci
PCA	Principal Component Analysis (PLS)
PLS	Partial Least-Squares Analysis (PLS)
RAM	Root Apical Meristem
ROS	Reactive Oxygen Species
RB	Right Border
SAM	Shoot Apical Meristem

SE(s)	Somatic Embryo(s)
SEM	Scanning Electron Microscopy
SOD	Superoxide Dismutase
SSA	Sonication-Assisted *Agrobacterium* mediated transformation
SSEO	Site of Somatic Embryo Origin
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid-Reactive-Substances
TDZ	Phenyl-N-(1,2,3-Thiadiazol-5yl) Urea
TEM	Transmission Electron Microscopy
v/v	Volume by Volume
w/v	Weight by Volume
XOD	Xanthine Oxidase
X-glu	5-Bromo-4-Chloro-3-indolyl–D-Glucopyranoside
Zeatin	6-(4-Hydroxy-3-Methyl-Trans-2-Butenylamino)Purine

CHAPTER 1

INTRODUCTION

1.1 Background

Maize is the most important cereal crop on a worldwide basis (Jones, 2009). Currently, maize is the most extensively cultivated cereal crop, followed by wheat and rice (Pechanova et al., 2013). It is grown in all continents with the exception of Antarctica. Presently, the United States is the largest producer as well as world's largest consumer of maize (Torney et al., 2007).

Conventional maize breeding is the art and science of compromise. Multi-trait selection, multi-stage testing, and multi-progeny evaluation are common for discarding thousands of lines and hybrids (Hallauer and Carena, 2009). It has been argued that the major drawback of traditional methods of maize breeding is to ascertain the genetic quality or excellence of line in hybrid. The majority of traits that are of economic significance are quantitatively inherited. Their importance is recognized by molecular geneticists through their emphasis in quantitative trait loci (QTL) experiments, molecular markers, marker-assisted selection to predict early and late generation combining abilities, and/or ultimately gene-assisted selection through specific genome selection (e.g. meta QTL analyses) and/or association mapping (Hallauer and Carena, 2009). In a nutshell, the prime goals of maize breeding are; germplasm improvement, development of pure lines, generation of hybrids between the derived lines, identification and selection of reliable hybrids with predictable agronomic behaviour and adaptation across diverse agricultural zones and finally generation of excellent variety for farmers's use (Hallauer and Carena, 2009). Despite the fact that these traditional approaches paid-off, the challenges still remain. Some of these challenges are, cost, time consuming and laborious.

Few decades ago, a more powerful, feasible and dependable approach was devised. This breakthrough was collectively termed as biotechnology- which is a collection of many technologies/techniques such as marker assisted breeding, genetic engineering, cell and tissue culture techniques and molecular cloning. All these are for the overall enhancement of plant/plant products. However, the success of genetic engineering in plants depends entirely on reliable, efficacious and reproducible tissue culture techniques. Even though zygotic embryo culture has been in practice since 1904 (Raghavan, 2003), the first report documenting tissue culture and plant regeneration in maize was communicated by Green and Philips (Green and Phillips, 1975). In this and other subsequent studies, it was shown that the regeneration was through somatic embryogenesis in friable type II callus. Type II callus is characterized by rapid growth, is very fragile and easily breakable, thus termed friable. Friable type II callus can be proliferated expeditiously by timely sub-culture. Type II callus is generally not easily induce in most maize germplasms and its differentiation is very minimal or near zero. The color of this callus ranges

from light yellow to white. On the contrary, type I embryogenic callus is characterized by high degree of differentiation. Type I callus is compact and grows very slowly (Ishida et al., 2007).

Successful regeneration of maize via somatic embryogenesis and organogenesis have been reported from different genotypes and different sources of explants. Immature embryos from maize have been considered as the explant of choice in many genotypes (Manivannan et al., 2010; Seth et al., 2012; Zhong et al., 2011). Endosperm culture from Hi II (Thomas and Chaturvedi, 2008; Moeller et al., 2012), immature tarsel (Grando et al., 2013), anther culture (Jäger et al., 2010), leaf (Ahmadabadi et al., 2007), mature embryo (Zhao et al., 2008; Jia et al., 2008), shoot apices (**?**Muoma et al., 2008). However, genetic transformation of maize was only possible during the early 90's when Gordon-Kamm and co-workers reported successful regeneration of transformed maize. (Gordon-Kamm et al., 1990).

Since then, various transformation techniques were invented. These includes electroporation of immature zygotic embryo or type I callus (D'Halluin et al., 1992), cell suspension cultures (Laursen et al., 1994), type II callus (Pescitelli and Sukhapinda, 1995). Interestingly, in the mid 90s another breakthrough technology in maize transformation was reported using *Agrobacterium* (Ishida et al., 1996). The first successful regeneration of maize transformed by silicon carbide whiskers was also achieved through cell suspension cultures (Frame et al., 1994). These and other several techniques are discussed in review literature thoroughly.

The formation of friable cultures and generation of transgenic maize plants are entirely dependent on so many factors such as culture media, type and source of explant, genotype among others (Songstad, 2010a). In all the afore mentioned factors, genotype was found to be most limiting as compared to others. It is pertinent to note that majority of successful work done or protocols that were established, were developed using the maize model genotype A188, B73 and/or their hybrid Hi-II (Che et al., 2006) or to a lesser extent H99 and Mo17 (Ishida et al., 2003; Frame et al., 2006b). High degree of embryogenic callus formation and regeneration are among the good attributes of these genotypes. However, these are generally regarded as poor lines from agronomical point of view. As such, the introduction of transgenes into local varieties is only feasible by back-crossing which is laborious and costly (Lupotto et al., 2004).

The maize inbred cultivars used in this research were obtained from the international maize and wheat development centre or simply CIMMYT; an acronym which in Spanish it stand for ***Centrino Internscional Mejoramiento de Maiz Y Trigo***. In brief, CML 406 and 419 have white grain and and are adapted to lowland.The former mature late while later matures early. CML 425 and 427 are yellow grain, have early maturity time and are all adapted to Asia low land. These four lines were chosen

because preliminary investigations shows they respond better tissue culture compared to the remaining sixteen lines. In addition, to the best of our knowledge, no previous work on tissue culture was reported on these inbred lines. Therefore this work was conducted to explore potentials of these lines for *in vitro* manipulations using tissue culture, genetic transformation and some histobiochemical examinations.

1.2 Research Problem

Maize is one of the most staple food consumed in Sub-Saharan Africa and Latin America. Global demand of this cereal crop is at increase due to increase in population growth and decrease income in house hold units in the affected areas. Thus new lines with superior quality are being developed. Traditional crop improvement alone is insufficient to meet the demand hence the need for genetic transformation. Genetic transformation of maize is dependent on the formation of reliable and efficient plantlet regeneration system. However, previously established protocols for tissue culture and genetic transformation are based on maize model genotypes such as A188, B73, Hi-II, H99 and Mo17. Hence there is a need to develop a new protocols for these new lines since it's a known fact *in vitro* cultures is majorly dependent on genotype.

1.3 Significance of the Study

Presently no available literature indicated any *in vitro* manipulation studies on this genotype. Therefore this study investigates the potentials of these maize lines for *in vitro* regeneration and genetic transformation study. Additionally, metabolomics studies on the key morphogenic pathways (somatic embryogenesis and organogenesis) was also investigated. These will no doubt add value to the existing knowledge in the field of *in vitro* of maize.

1.4 Objectives of the Study

The objectives of this research are:

i. To establish callus initiation, maintenance, and regeneration of CML 419 and CML 427 maize lines.

ii. To investigate morphohistological features of the resulting calli generated from CML 419 and CML 427.

iii. To characterize biochemical changes in embryogenic and organogenic calli from these lines.

iv. To establish a predictable high efficiency *Agrobacterium tumefaciens* mediated transformation protocols for CMLs 419 and 427 maize lines.

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