UNIVERSITI PUTRA MALAYSIA

IN VITRO CULTURE OF MAIZE (ZEA MAYS L.) INBRED LINE SM5-4

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FBSB 2005 20

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of

APRIL 2005

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

IN VITRO CULTURE OF MAIZE (_Zea mays_ L.) INBRED LINE SM5-4

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April 2005

Chairman: Associate Professor Norihan Mohd. Saleh, Ph.D.

Faculty: Biotechnology and Biomolecular Science

Tissue culture of inbred line SM5-4 maize (*Zea mays* L.), maternal parent of Putra J-58 (F_1 hybrids) was established using maize zygotic embryo as explant. To obtain embryogenic callus from mature and immature zygotic embryos of inbred line SM5-4, manipulations of media components such as carbon, nitrogen, proline, and casein hydrolysate, and culture conditions such as incubation temperature, light, were carried out. Immature embryos have the ability to form callus and embryogenic calli, which can result in plant regeneration.

At tissue culture level, the study aims at establishing the best tissue culture system via somatic embryogenesis and to overcome plant regeneration problems by manipulating the sucrose concentration, hormone combination and concentration, culture age, the type of medium formulation used to grow callus, incubation temperature, light and media formulation.

Sterilization technique of maize from mature and immature maize seeds of the inbred line SM5-4 was investigated. Mature seeds (50 days old) and immature seeds (14 days after pollination) were disinfected by washing in different concentrations of sodium hypochlorite (Clorox) for different duration. Disinfection in 50%(v/v) Clorox solution (2.27% sodium hypochlorite) for 20 minutes gave 90% of contamination-free culture of mature seeds whereas 50%(v/v) Clorox solution (2.27% sodium hypochlorite) for 15 minutes gave 75% of contamination-free culture of immature seeds. Reduction in Clorox concentration of 20% (v/v) Clorox (1.05% sodium hypochlorite) for 20 minutes gave high percentage (67%) of contamination- free culture of immature seeds that remain viable.

N6 basal medium was found to be the best medium in enhancing both callus induction and embryogenic calli formation. The highest callus induction frequency on N6 basal medium supplemented with 9 μM 2,4-D from immature zygotic embryos was 79.5%. Both plants growth regulators, 2,4-D, IAA and BAP, kinetin, were capable of switching on the induction of callus necessary for embryogenic totipotency. The combination of 2,4-D and kinetin were however more effective in producing callus induction in embryos culture of maize.

The most effective method for producing friable, embryogenic callus was found for immature zygotic embryos. Maturation of somatic embryos was enhanced by transferring the embryogenic callus after 4 weeks to medium containing 6% sucrose and 1mg/L NAA. During the following 3-4 weeks, as the somatic embryos developed, the cultures were transferred to the regeneration medium (MSO). Approximately 80% of immature zygotic embryos produced embryogenic callus and

then plantlets. Immature embryos of inbred line SM5-4 produced the highest percentage of callus and showed the highest number of plant regeneration compared to mature zygotic embryos.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PENGKULTURAN *IN VITRO* KULTUR UNTUK JAGUNG (*Zea mays* L.) TITISAN INBRED SM5-4

Oleh

NGUYEN THI MAI ANH

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Kultur tisu jagung (*Zea mays* L.) titisan inbred SM 5-4, kacukan daripada induk variasi hibrid-hibrid F_1 yang dikeluarkan oleh Putra J-58 telah dilakukan. Pengkulturan dilakukan secara *in vitro* menggunakan embrio zigotik jagung sebagai eksplan kultur untuk menghasilkan kalus embriogenik daripada embrio zigotik titisan inbred SM 5-4 yang matang dan yang tidak matang, dengan memanipulasi komponen-komponen media seperti karbon, nitrogen, prolin dan kasein hidrolisat; serta keadaan kultur seperti suhu pengeraman dan cahaya. Embrio-embrio yang tidak matang mempunyai kebolehan untuk menginduksi kalus dan membentuk kalus embriogenik yang membolehkan regenerasi tumbuhan berlaku.

Di peringkat kultur tisu, kebanyakan kajian yang dijalankan adalah bertujuan untuk mendapatkan sistem kultur tisu terbaik melalui embriogenesis somatik dan mengatasi masalah regenerasi tumbuhan. Dengan memanipulasikan kepekatan sukrosa,

kombinasi dan kepekatan hormon, usia kultur, formula medium yang digunakan untuk pembentukan kalus, suhu pengeraman, cahaya dan formulasi media.

Teknik pensterilan kultur jagung daripada benih titisan inbred SM 5-4 matang dan yang tidak matang juga dikaji. Benih yang matang iaitu berumur 50 hari, manakala benih yang tidak matang berumur 14 hari selepas pendebungaan. Benih-benih itu disteril dengan pelbagai kepekatan dan tempoh cucian menggunakan sodium hipoklorik (Clorox). Cucian 50% (Isipadu/Isipadu) Clorox (2.27% sodium hipoklorik) selama 20 minit memberi bacaan keputusan 90% bebas kontaminasi benih matang setelah dinyahkumankan dengan sepenuhnya. Manakala, cucian 50% (Isipadu/Isipadu) Clorox (2.27% sodium hipoklorik) selama 15 minit memberi keputusan bebas kontaminasi dalam benih yang tidak matang sehingga 75%. Pengurangan kepekatan Clorox sehingga 20% (Isipadu/Isipadu) (1.05% sodium hipoklorik) untuk pensterilan selama 20 minit memberi 67% bebas kontaminasi dalam benih yang tidak matang, yang masih kekal hidup.

Medium basal N6 didapati merupakan medium terbaik dalam penghasilan kedua-dua induksi kalus dan kalus embriogenik. Kekerapan maksima untuk induksi kalus daripada zigotik embrio yang tidak matang dalam media basal N6 ditambah dengan 9 µM 2,4-D adalah sebanyak 79.5%. Kedua-dua hormon pengawal pertumbuhan iaitu auksin (2,4-D, IAA) dan sitokinin (BAP, Kinetin) adalah sesuai bagi induksi kalus, bersedia untuk totipotensi embriogenik. Kombinasi 2,4-D dan Kinetin merupakan kombinasi yang lebih berkesan dalam menghasilkan induksi kalus dalam kultur embrio jagung.

Keberkesanan yang paling tinggi dalam menghasilkan kalus embriogenik terbaik dibentuk oleh zigotik embrio yang tidak matang. Kematangan embrio somatik dapat dipercepatkan dengan memindahkan kalus embriogenik dalam kultur selepas 4 minggu ke medium yang mengandungi 6 % sukrosa dan 1 mg/l NAA. Dalam tempoh masa 3 ke 4 minggu kemudian, pembentukan embrio somatik berlaku. Embrio somatik itu perlu dipindahkan ke medium regenerasi iaitu MSO. Sejumlah 80% embrio zigotik yang tidak matang boleh menghasilkan kalus embriogenik dan kemudian membentuk pokok plantlet. Keputusan menunjukkan embrio titisan inbred SM 5-4 yang tidak matang menghasilkan peratusan kalus dan kebolehan regenerasi tumbuhan yang tertinggi berbanding embrio zigotik yang matang.

ACKNOWLEDGEMENTS

No words could describe my heartfelt gratitude and appreciation to Assoc. Prof. Dr. Norihan Mohd. Saleh for her constant guidance and invaluable advice. Her constant motivation and words of advice, has inspired me to become a better researcher and individual.

Special thanks are extended to other members of my supervisory committee, Prof. Dr. Ghizan Saleh and Dr. Mohd Azmuddin Abdullah for their advice, comments and guidance whenever sought.

My appreciation is also extended to the tissue culture lab. members especially Kak Fadilah, Kak Lisa. I would like to thanks Kak Fuziah, Mr. Ong and Plant Breeding lab members of the Faculty of Agriculture, especially Kak Maininah, Udin, Ros and Nor for all their help and support.

Lastly, but not least, to my friends, to those whom I have stumbled upon somewhere and sometimes, to all, named and unnamed – I present this thesis as testimony of each and everyone's unique contribution.

I certify that an Examination Committee met on 8th April 2005 to conduct the final examination of Nguyen Thi Mai Anh on her Master of Science thesis entitled "*In vitro* Culture of Maize (*Zea mays* L.) Inbred Line SM5-4" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledge. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

NGUYEN THI MAI ANH

Date: 7 JUN 2005

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
BAP	6-Benzylaminopurine
^{O}C	degree centigrade
CIMMYT	Centro Internacional de Mejoramiento de Maiz Y Trigo International Maize and Wheat Improvement Center
d	day
2,4-D	2,4-dichlorophenoxy acetic acid
dH_2O	distilled water
EC	embryogenic calli
g	gram
h	hour (s)
IAA	Indole-3-acetic acid
K	6-Furfurylaminopurine (kinetin)
L	liter
M	Molar
mM	milliMolar
ml	milliliter
mg	milligram (s)
min	minute
MS	Murashige and Skoog
MSO	hormoneless Murashige and Skoog
µ l	microliter
µM	micromolar
NAA	napthylacetic acid
NEC	non-embryogenic cells
%	percentage
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Maize (Zea mays L.) or commonly called corn is probably the third most widely grown crop in the world, after wheat and rice (Kiesselbach, 1999). It was first discovered by the Europeans in 1492 and was spread all over the world, following the first voyage of Christopher Columbus to America at the end of the 15th century (Jeffs, 1986). Since then, it has made a great economic significance worldwide as human food, animal feed and as a source for a large number of industrial products. Maize has multifarious uses and the only cereal whereby various stages of plant development has utilities. Young maize ear shoots ("baby corn") can be used as vegetables and harvested as soon as the plant flowers. The tender green ears of sweetcorn are delightful delicacies. The maize plant, which is still green when ears are harvested as baby ears or green ears, makes good forage. Besides being served as human staple food, maize can be used as livestock feed. It has the highest net energy content and lowest content of protein and fiber, which is preferable for animals.

Maize is a member of the Graminae or grass family, placed in the tribe Maydeae. It is monocotyledonous, with a diploid chromosome number of 2n= 2x=20 (Bajaj, 1990). Maize is a particularly convenient plant for genetic studies because it can readily be either self or cross-pollinated with large numbers of seeds per plant. Hence, work with maize is easily justified by the potential value of any new approaches towards improvement of the yield or its resistance against pests or diseases.

Like other cereal crop, maize varieties have been subjected to extensive investigation in the areas of plant tissue culture and biotechnology, especially on callus induction and somatic embryogenesis. Somatic embryogenesis is the process of obtaining embryos from somatic plant tissues. Many species of monocotyledons particularly the Graminae have been shown to form somatic embryos in cultures derived from young leaves, inflorescences, and immature embryos (Hakman et al., 1985). Somatic embryogenesis may be the principal pathway for plant regeneration in tissue cultures of important group of crops plants (Vasil, 1982; 1983). The successful plant regeneration from protoplasts culture is dependent on the genotypes and the age/stage of the culture (Morocz et al., 1990). Protoplasts isolated from several genotypes of maize viz. A188 (Rhodes et al., 1988), Cat 100-1 (Prioli and Sondahl, 1989), HE / 89 (Morocz et al., 1990) and B73 (Shillito et al., 1989) have been reported to regenerate into complete plants. Morocz et al. (1990) reported that more than 90% of the regenerated plants survived transplantation to soil and developed into plants of normal appearance. Analysis of large number of the lines and hybrids of maize demonstrates that the ability to induce callus and plant regeneration is determined to a considerable extent by the genotype of the initial plant (Nesticky et al., 1983; Tomes and Smith, 1985; Chernyskova et al., 1988). Comparison of calli induction from tissue of maize heterotic hybrids and their parental forms: Svetoch (VIR 40 x VIR 43), Salva (VIR 44 x VIR 38), Karasnodarsky 303TV (64 x Ci 25), Iskra (VIR 26 x VIR 27) and Kharkovsky 10T (Starinskaya x Kharkovskaya 46) showed that the initiation and growth of calli from explants of maize hybrids are more rapid than from their parental lines (Ivantsov et al., 1994). In addition, growth of calli from maternal tissue is more rapid than the paternal tissue (Ivantsov et al., 1994). In many ways, maize is the flowering plant best suited for biological research

on gametes (Freeling and Walbot, 1993), fertilization and embryogenesis (Dumas and Mogensen, 1993). Research on callus initiation and somatic embryogenesis in maize can lead to a better understanding of in vitro cultures, particularly for maize and possibly for monocotyledons in general. Studies on somatic embryogenesis have contributed a great deal of information on genetic, morphological and physiological changes during zygotic embryo formation in vivo and early embryo development. Somatic embryos may be used as a convenient way to propagate large numbers of genetically identical plantlets. As somatic embryos are bipolar, they readily possess root and shoot axes, which give rise to these organs in vitro. Only limited number of cells could form somatic embryos at any one time, and the fraction is highly variable among plant species. It depends in part on the genotype of the plant and the source of the explant (Nesticky et al., 1983).

Sweet corn (Zea mays L. saccharata) cultivation in Malaysia utilizes both local open-pollinated and imported hybrid varieties (Saleh et al., 2001). However the imported hybrid varieties are less adaptable to the local environment. Breeding efforts are therefore necessary to reduce the cost of importing corn seeds, and to produce sweet corn varieties that are of high yielding, high quality, edible and adaptable to local environmental conditions. Maize Putra J-58 variety is the first grain maize hybrid variety ever being released in Malaysia. The breeding programme for the variety was conducted from 1987 until 1998 and the variety was launched on 8th October 1998. Breeding of Putra J-58 was carried out at the Department of Agronomy and Horticulture, Faculty of Agriculture, Universiti Putra Malaysia. It is an F1 hybrid produced from a cross between two superior inbred lines, i.e. SM5-4 x SW9 (Saleh, 1998). This grain maize variety is most suitable for use as animal feed due to its high

grain yield, uniform plants and reasonably early in maturity, as compared to the open-pollinated varieties available in the country, tolerant to environmental constraints and has high nutritive values (Saleh, 1998). Selection of superior inbred lines for their combining abilities for hybrid production demands a great amount of effort. For successful hybrid variety development, heterotic effects have to be maximized and the best results are expected when two unrelated or diverse inbred lines are used. This study was carried out as part of the inbred line maize parent (inbred line SM5-4), where characters of these parental inbred lines are an important consideration for transferring hybrid F1 variety (Putra J-58). These parental inbred lines are important factors in determining combining ability among female inbred line and heterosis revealed by the F1 hybrid, where a dominant gene is expected to enter into a genetic transformation.

Extensive investigations on maize tissue culture have suggested that the tissue culture responses of maize explants are genotype-specific. Genotype strongly affects culture responses with some lines producing embryogenic callus on virtually all cultured embryos, and others giving either little proliferation or only non-regenerable callus (Lu et al., 1982). In maize, hybrid varieties (F1) are characterized by high yield and uniformity of the plants. It is an expression of heterosis or hybrid vigour revealed by the inbred parents (CIMMYT, 1988). Response of different maize lines in culture is heritable, and crosses of regenerable lines with poorly regenerable ones often give F1 progeny with improved cultivability (Beckert and Qing, 1984).

There is no report yet on the optimization of media and culture conditions for tissue culture by maize inbred line SM5-4 zygotic embryo cultures. Therefore, the objectives of this research are:

i). To establish tissue culture procedure for maize inbred line SM5-4 from mature and immature zygotic embryos.

ii). To optimize callus growth and somatic embryogenesis of SM5-4 through the manipulation of medium composition and environmental conditions.

iii). To determine the plant regeneration efficiency of maize inbred line SM5-4 from mature and immature zygotic embryos.

CHAPTER 2

LITERATURE REVIEW

2.1 Classification and Taxonomy of Maize

Apart from maize (*Zea mays* L.), other relatives of maize include *Teosinte* and *Tripsacum,* which are important wild relatives of *Zea mays*. These may provide important possible sources of desirable traits for the continued improvement of maize. *Teosinte* and maize cross freely and any genes for resistance and tolerance to natural stresses in *Teosinte* should have already been transferred to maize (Galinat, 1988). Wilkes (1989) believes that the history of *Teosinte* transformation into maize with the power of human selection, opens up immense possibilities for the use of existing and new genetic variability in *Teosinte* populations for creative maize breeding. *Teosinte* is similar in general appearance to maize, which has an apical male part (seed-bearing), female parts along branches of the stem (Figure 1). The main difference is that maize produces a few large ears with many seeds, while *Teosinte* produces a vegetative shoots and the ears are tiny in comparison (Figure 1) (Chrispeels, 2003). *Tripsacum*, the other wild relative of maize, does not hybridize freely with *Teosinte* or maize, but it is the only genus with which maize has been crossed and viable hybrids produced and grown to maturity (James, 1979). In the whole tribe of *Maydeae*, maize is the only cultivated species of great economic importance, while *Tripsacum* has no direct economic value, and *Teosinte* is used as a source of fodder.

Figure 1. Maize (left) in close proximity to *Teosinte* (right) (Chrispeels, 2003).

There are five major types of maize - dent, flour, flint, popcorn and sweet (Jeffs, 1986). Dent maize (*Zea mays* var. indentata), is the most widely grown type of maize in the United States and Northern Mexico. Dent maize is a cross between flint and floury maize. Soft starch extends to the kernel apex but shrinks on drying to produce the "dent". Floury maize (*Zea mays* var. amylacea), is one of the oldest types of maize and is characterized by the lack of any hard or vitreous endosperm. It is widely grown in the dry areas of the United States and in the South Africa. The kernels are almost entirely soft starch. Flint maize (*Zea mays* var. indurata) has a thick, hard vitreous endosperm, which surrounds a small center of soft or floury endosperm. It is mostly grown in Europe, Asia and Central and South America. The kernels contain little starch. Popcorn maize (*Zea mays* var. tunicata) is an early variation of flint maize, which has been selected over the years for the ability of the kernels to pop during rapid cooking. Not grown commercially, each kernel is enclosed in a pop or husk. Sweet corn (*Zea mays* var. saccharata), is a genetic variant maize, which inhibits the conversion of sugar into starch during kernel filling. It differs from dent maize only by one recessive gene that prevents conversion of some of the sugar into starch (Wolfe *et al.*, 1997). Hybrid cultivars of sweet corn are widely adopted in Europe and North America; open-pollinated cultivars are still used in these areas and elsewhere.

2.2 Pollination of Maize Plant

Maize has the basic structure of the grass family with conspicuous nodes and internodes on the stem. It is a monoecious plant, which means that it has separate male and female inflorescences on the same plant. The female inflorescence, the ear, arises from the axillary apiece. The male inflorescence, the tassel, develops from the