UNIVERSITI PUTRA MALAYSIA

PRODUCTION, SELECTION, CHARACTERIZATIONS AND SOMATIC EMBRYOGENESIS OF MALAYSIAN SALT-TOLERANT RICE (Oryza sativa L. cv. MR219) THROUGH CALLOGENESIS

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By

NAHID KALHORI

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

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This thesis is dedicated to:

My parents for their endless love and support &
my brother for his encouragement

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

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Chairman : Rosimah Nulit, PhD
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Rice is one of the most important staple foods for human. However, millions hectares of land in the South and Southeast Asia were left uncultivated or grown with very low yields due to salinity. Therefore, the main objective of this study is to produce salt-tolerant Malaysia *Indica* rice cv. MR219) lines *in vitro*. The phenotypes, biochemical contents and seed germination capability of mother plant and salt-tolerant MR219 lines were compared. Firstly, MR219 callus was produced, then, callus was cultured separately on MS media supplemented with 2 mg/L 2,4-D and different concentration of NaCl (0, 50, 100, 200, and 300 mM NaCl) to produce salt-tolerant MR219 callus. Morphological comparison shows that MR219 callus from MS media yellowish color, soft, friable and nodular proliferating, however, callus produced in 100 mM NaCl are compact-type and blackish-brown and acutely-necrotic at 300 mM NaCl. Fresh and dry weight, water content, growth rate of MR219 callus and total protein content decreased as concentration of NaCl increased. On other hand, total proline content, total soluble sugar, lipid peroxidase and the activity of ascorbate peroxidase and catalase were increased. Histological analysis of salt-tolerant MR219 callus revealed that salinity negatively affected on development somatic embryos. Callus from 50 mM and 100 mM NaCl had been selected as salt-tolerant callus and was cultured on MS media supplemented with 2 mg/L kinetin and 1 mg/L BAP for shoot induction. Then, callus was subcultured in MS media supplemented with 0.5 mg/L BAP, 1 mg/L kinetin, 1 mg/L IBA and 0.5 mg/L NAA for root formation. At acclimatization stage, only MR219 plantlets from control (MS only) and 50 mM NaCl were survived and transferred to paddy soil. MR219 plantlets produced from 50 mM NaCl is called First generation (F1) salt-tolerant MR219. After 70 days, seeds of F1-salt-tolerant MR219 lines was successfully obtained. Following this, the grain characteristics of mother plant and F1-salt-tolerant MR219 lines were compared. Comparative study on phenotyes, leaves morphology, and root system found no variation between mother plant and second generation (F2)-salt-tolerant MR219 lines. Biochemical contents which are proline content, total soluble sugar and total protein showed no significant difference between mother plant and F2-salt-tolerant MR219 line. Seeds of F1-salt-tolerant MR219 was examined its germination capability in saline. Results found that seeds of F1-salt-tolerant MR219 able to germinate and growth in 50 mM and 100 mM NaCl. As conclusion, salt-tolerant MR219 rice was produced *in vitro* and have potential to be commercialized. The protocol to produce salt-tolerant rice can be used to produce other salt-tolerant plant.

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

PENGHASILAN, PEMILIHAN, PENCIRIAN DAN SOMATIK EMBRIOGENESIS PADI MALAYSIA YANG BERTOLERANSI DENGAN KEMASINAN MELALUI KALOGENESIS

Oleh

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Padi merupakan makanan ruji utama manusia. Walaibagaimanapun berjuta-juta kawasan penanaman padi di Selatan dan Asia Tenggara terbiar dan pengeluaran padi semakin berkurangan disebabkan oleh kemasina. Kajian ini dilakukan bertujuan untuk menghasilkan padi (*Oryza sativa* cv. MR219) yang toleran dengan kemasinan secara *in vitro.* Kajian ini bermula dengan penghasilan kalus MR 219, kemudian kalus ini dikultur secara berasingan pada MS media yang mengandungi 2 mg/L 2,4-D dan kepekatan NaCl yg berbeza (0, 50, 100, 200, and 300 mM NaCl) untuk mehasilkan MR219 kalus yang bertoleransi dengan kemasinan. Kajian perbandingan ke atas morfologi, fisiologi, kandungan biokimia dan histologi diantara kalus MR219 (dari MS sahaja) dan kalus MR219 yang toleran dengan kemasinan dilakukan. Kalus MR219 tanpa rawatan kemasinan menunjukkan warna putih kekuningan, lembut, rapuh dan berbentuk nodular. Walaubagaimanapun, kalus yang tumbuh daripada 100 mM NaCl adalah padat dan berwarna coklat-kehitaman, akut-nekrotik pada 300 mM NaCl. Kajian juga mendapati berat basah dan kering, kandungan air, kadar pertumbuhan kalus MR219 dan kandungan protin menurun dengan meningkatnya kepekatan NaCl. Manakala, kandungan prolina, kandungan gula terlarut, aktiviti lipid peroksidase dan askorbat peroksidase kalus MR219 meningkat dengan bertambahnya kepekatan NaCl. Aktiviti enzim katalase bertambah pada kalus yang toleran pada 50 mM dan 100 mM NaCl. Analisis histologi ke atas kalus MR219 yang toleran dengan kemasinan mendapati saiz zon meristematik berkurang. Kalus yang tumbuh pada 50 mM dan 100 mM NaCl telah dipilih sebagai kalus yang toleran terhadap kemasinan. Seterusnya, kalus ini disubkultur pada MS media yang diperkaya dengan 2 mg/L kinetin, 1 mg/L dan 0.5 mg/L NAA untuk pertumbuhan pucuk. Diikuti dengan subkultur pada MS media yang ditambah 0.5 mg/L BAP, 1 mg/L kinetin, 1 mg/L IBA dan 0.5 mg/L NAA selama 4 minggu untuk pembentukan akar. Di peringkat aklimatasi, hanya anak-anak padi MR219 yang terhasil dari kawalan (MS sahaja) dan 50 mM NaCl berupaya untuk membesar dan dipindahkan ke pot yang mengandungi tanah sawah untuk kajian seterusnya. Anak-anak padi MR219 yang toleran dengan 50 mM NaCl dinamakan sebagai generasi pertama (F1) padi MR219 yang toleran dengan kemasinan. Selepas 70 hari, bijibenih F1 padi MR219 yang toleran dengan kemasinan berjaya dituai. Kajian perbandingan ke atas ciri-ciri morfologi pokok, morfologi daun dan jenis sistem akar generasi kedua (F2) padi MR219 yang toleran dengan kemasinan adalah sama dengan pokok induk. Kandungan biokimia iaitu prolina, kandungan gula larut dan protein menunjukkan tidak signifikan (t-test, $p > 0.05$) antara pokok induk dan F2-padi MR219 yang toleran dengan kemasinan. Bijibenih F1 padi MR219 yang toleran dengan kemasinan berupaya untuk bercambah dan membesar dalam 50 mM dan 100 mM NaCl. Sebagai kesimpulan, padi MR219 yang toleran kepada kemasinan berjaya dihasilkan secara *in vitro* dan berpotensi untuk dikomersialkan. Protokol penghasilan padi yang toleran dengan kemasinan ini boleh digunapakai untuk penghasilan tumbuhan lain yang toleran dengan kemasinan.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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LIST OF ABBREVIATIONS

APX	Ascorbate peroxidase
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
CAT	Catalase
Cl^-	Chloride ion
CO_2	Carbon Dioxide
dSm^{-1}	DeciSiemens per meter
DW	Dry weight
EC	Electrical conductivity
ESP	Exchangeable sodium percentage
FAO	Food and agriculture organization
FW	Fresh weight
GR	Glutathione reductase
H^+	Hydrogen ion
HCO_3	Bicarbonate
H_2O_2	Hydrogen peroxide
ddH_2O	Distilled water
DNA	Deoxyribonucleic acid
K^+	Potassium ion
kcal	Kilocalorie
KCl	Potassium chloride
M	Molar
MARDI	Malaysian agriculture research & Development institute
Mha	Million hectares
MR	Malaysian rice
mg	Milligram
mg/g	Milligram per gram

mg/L	Milligram per litre
Mg^{2+}	Magnesium ion
$MgSO_4$	Magnesium sulphate
ml	Millilitres
mM	Mili molar
µl	Micro Litter
Na^+	Sodium ion
NaCl	Sodium chloride
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced Nicotinamide Adenie Dinucleotide Phosphate
Na_2SO_4	Sodium sulphate
NO_3^-	Nitrate
O_2^{2-}	Superoxide
$^1O_2$	Singlet oxygen
O^-	Superoxide radicals
OA	Osmotic adjustment
$OH^\bullet$	Hydroxyl radicals
pH	Potential hydrogen
QACs	Quaternary ammonium compounds
ROS	Reactive oxygen species
SE	Standard error
SO_4^{2-}	Sulphate
SOD	Superoxide Dismutase
UK	United Kingdom
UPM	Universiti Putra Malaysia
USA	United States of America
USDA	United States Department of Agriculture
USSR	Union of Soviet Socialist Republics

UV	Ultraviolet
V	Voltage
X	Time
°C	Degree Celsius
%	Percentage

CHAPTER 1

INTRODUCTION

1.1 Background of study

Salinity as a major environmental constraint to crop productivity commonly occurs in arid and semiarid regions (Shrivastava & Kumar, 2015). Under high salt stress conditions, most of the crop plants are susceptible and unable to survive. Increased salinization in coastal areas and arable land is predicted to become a huge problem throughout much of the world. Approximately 6.5% of world's total area and about 20% of the cultivated area has already been affected by soil salinity. All over the world, about 397 million ha of land have been affected by different types of salts such as sodium chloride (NaCl), calcium chloride ($CaCl_2$), sodium sulphate (Na_2SO_4), and magnesium sulfate ($MgSO_4$) (Diédhiou, 2006). These type of salts are highly soluble into water and releasing salt ions such as Na^+, Ca^{2+}, Mg^{2+}, Cl^- and SO_4^{2-} (Hakim *et al.*, 2014).

Crop plants exhibit a spectrum of reactions against salinity. Salt stress has two primary harmful effects; osmotic and ionic stress (Diédhiou, 2006). Osmotic stress leads to reduction of water uptake by root, and accumulation and toxicity of specific ions caused ionic stress. Both ionic and osmotic stresses lead to reduced growth rates and eventually to plant death. Followed by primary stresses, oxidative damage as secondary stress may occur (Gupta & Huang, 2014). Limited CO_2 fixation because of stress conditions leads to a decrease in (1) carbon reduction by Calvin cycle and (2) oxidized NADP to serve as an electron acceptor in photosynthesis (Roach & Krieger-Liszkay, 2014). When ferrodoxin is over reduced during photosynthetic electron transfer, electrons may be transferred from photosystem (PS)-I to oxygen to form superoxide (O^{2-}_2) radicals by Mehler reaction (Khan *et al.*, 2015) which triggers chain reactions that generate more aggressive oxygen radicals containing $^1O_2$, H_2O_2, $O^{\bullet -}_2$, and $OH^{\bullet}$, which are known as reactive oxygen species (ROS).

Plants have developed complex mechanisms to adjust hyperosmotic stress and ionic imbalance by osmotic adjustment (OA). These mechanisms accumulate osmotic regulators such as sugars and proline to protect membrane integrity and stabilize enzymes against oxidative stress (Manai *et al.*, 2014). Meanwhile, to overcome the harmful effects of ROS, plants developed non-enzymatic and enzymatic antioxidant defenses systems. A non-enzymatic defense system involves phenolic compounds and lipid peroxidation. An enzymatic defense system include catalase (CAT; E.C.

1.11.1.6), and ascorbate peroxidase (APX; E.C. 1.1.1.11) (Abogadallah, 2010; Sharma *et al.*, 2012; Ismail *et al.*, 2014).

1.2 Problem Statement

Glycophytes such as rice are very sensitive to saline soil especially at the early stage of growth, with height, root length, emergence of new roots, and dry matter affected significantly by salinity (Pearson *et al.*, 1966; Akbar *et al.*, 1972). Rice has no various strategies and mechanisms to deal effectively with the excessive presence of salt and therefore does not grow well on saline soil (Galvan-Ampudia & Testerink, 2011). Salinity caused a negative impact on a number of yield components including stand establishment, panicles, delayed flowering, tillers and spikelets per plants, floret sterility, and individual grain size and even delayed heading (Kar & Shaw, 2013). Under salinity condition, rice yields decrease 12% for every unit (dS/m) increase in EC_e (average root-zoon EC of saturated soil extract) above 3.0 dS m^{-1} (Maas & Hoffman, 1977).

The need for the development of salt tolerant rice is well documented (Flowers & Yeo, 1995). Breeding programme for salt tolerance in rice is difficult due to the involvement of several genes and insufficient knowledge about mechanism (s) controlling the characters (Yeo *et al.*, 1990). The other important issue that the increasing demand of rice consumers in the 21st century cannot be met only by traditional breeding efforts. In order to ensure the food security, plant cell and tissue culture techniques are being used for the genetic improvement and developing salt tolerant lines of rice throughout the world. Tissue culture techniques elucidate the cellular mechanisms involved in salt tolerance by using as study system *in vitro* selected NaCl tolerant cell lines (Davenport *et al.*, 2003; Gu *et al.*, 2004). Cell lines with enhanced tolerance to NaCl have been isolated from crop plants and various biochemical processes appear to contribute to the adaptation of cells to salinity. Besides the use of tissue culture in selection of salt tolerant cell lines, these lines have been used to regenerate salt tolerant plants (Shankhdhar *et al.*, 2000; Miki *et al.*, 2001). Several researches has developed salt tolerant plants especially using *in vitro* selected NaCl tolerant rice cell lines (Lutts *et al.*, 1999; Ahmad *et al.*, 2007; Khaleda *et al.*, 2007; Tariq *et al.*, 2008; Evangelista *et al.*, 2009; Rattana & Bunnag, 2015) and a wide range of plant species including cereals, vegetables, fruits and other commercially important plant species such as cauliflower (Elavumoottil *et al.*, 2003), sugarcane (Badawy *et al.*, 2008), sunflower (Alvarez *et al.*, 2003), lemon (Piqueras *et al.*, 1996), potato (Sabbah & Tal, 1990; Queiros *et al.*, 2007), and wheat (Zair *et al.*, 2003).

1.3 Objectives of Study

Malaysia *Indica* rice (*Oryza sativa* L. cv. MR219) was a cross resulted between MR137 and MR151 rice varieties which was produced by the Malaysian Agriculture Research and Development Institute (MARDI) in year 2001 (Panjaitan *et al.*, 2009). This rice variety is considered as high yielding rice and has good quality in term of shape and taste, but it is sensitive to environmental changes. According to Bot *et al.* (2000), land area in Malaysia is salt affected which restricted high production of rice. Therefore, this rice has been chosen in the present study.

Thus, the objectives of study are:

1. To produce, screen, select and regenerate first generation (F1) salt-tolerant MR219,
2. To compare the histological changes of MR219 callus under NaCl treatment,
3. To compare the histological changes of root structure between mother plant and F1-salt-tolerant MR219,
4. To compare the phenotypes and biochemical contents (total proline, total soluble sugar and total protein) between mother plant and F2-salt-tolerant MR219, and
5. To evaluate the germination capability of seeds of F1-salt-tolerant MR219 in NaCl solution.

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