UNIVERSITI PUTRA MALAYSIA

PLANT REGENERATION AND BIOCHEMICAL CHANGES IN RICE (*Oryza sativa* L.) UNDER NaCl STRESS

MOHAMMAD SAEED KHAN KHATTAK

FSAS 1996 13

PLANT REGENERATION AND BIOCHEMICAL CHANGES IN RICE (*Oryza sativa* L.) UNDER NaCl STRESS

BY

MOHAMMAD

Dissertation Submitted in Fulfilment of the Requirements
for the Degree of Doctor of Philosophy
in the Faculty of Science and Environmental Studies
Universiti Pertanian Malaysia

October, 1996

In the Name of Allah, the Most Gracious, Ever Merciful.

Dedicated To:

My respected parents and my wife and children.

ACKNOWLEDGMENTS

All praise belongs to **Almighty Allah**, **ever Merciful,** the **most Beneficent**. Has it not been due to **His** will and favour, the completion of this study would not have been possible.

I would like to express my deepest gratitude, honour and sincere appreciation to my chairman, Professor Dr. Marziah Mahmood for her invaluable guidance, encouragement and constructive suggestions throughout my study period. Her enthusiasm and patience have left a feeling of indebtedness which cannot be fully expressed.

My heartfelt appreciation and gratitude go to the member of my supervisory committee, Associate Professor Dr. Mohammad Arif Syed for his invaluable advice, guidance, encouragement and time throughout the research project and for reviewing the manuscript and making valuable comments from time to time.

I extend my sincere gratitude to the member of my supervisory committee, Dr. Radzali Muse for his generous guidance and encouragement throughout this study.

My deep appreciation is accorded to the Head and all staff members of Department of Biochemistry and Microbiology for their kind cooperation and warm behaviour.

I also extend my sincere appreciation to the Dean and all the staff members of the Faculty of Science and Environmental Studies for their kind cooperation and help.

I am grateful to Malaysian Technical Cooperation Programme (MTCP) for awarding me the fellowship to pursue this graduate study. Special thanks are to Mr. Adnan of MTCP for his cooperation. Gratitude is expressed to the Biotechnology Department of Malaysian Agricultural Research and Development Institute (MARDI) and Pakistan Agricultural Research Council (PARC), Islamabad for providing rice seeds to conduct this study.

I take pleasure to thank all my friends for providing very pleasant environment during my study at UPM. Mr. Jualang Azlan Abdullah is credited for the Bahasa Malaysia translation of the abstract. My regards are due to Mr. Abdul Aziz and Mdm. Faridah Shamsuddin of Graduate School for their kind cooperation throughout this study.

Finally, I would like to express my indebtedness to my father, mother, father in law, mother in law, brothers, sisters and sisters in law for their moral support, affection and encouragement in all my undertakings. Last, but not the least, the patience, endurance, love, sacrifices and understanding of Robina Khan, Seema, Kulsoom, Bilal, Owais, Ali and Haroon who helped me to complete this work.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

F. Wt	fresh weight
D. Wt	dry weight
SCV	settled cell volume
2,4-D	2,4-dihlorophenoxyacetic acid
BA	benzyladenine
NAA	naphthalene acetic acid
IAA	indoleacetic acid
K	kinetin
PP	Puteh Perak
B-370	Basmati-370
Mah	Mahsuri
NB	Nona Bokra
KG	Khari Gunja
GDH	glutamate dehydrogenase
GS	glutamine synthetase
GOGAT	glutamate synthase
NR	nitrate reductase
ACP	acid phosphatase
ALP	alkaline phosphatase
MDH	malate dehydrogenase
NADP-ICDH	NADP-isocitrate dehydrogenase
SDH	succinic dehydrogenase
SD	standard deviation
NaCl-T	NaCl-treated
C	control
g	gram
x g	times gravity
M	Molar
mM	millimolar

μM	micromolar
mg	milligram
min	minute
ml	milliliter
mmole	millimole
μmole	micromole
*n*mole	nanomole
M	Magnification for pictures

Abstract of Dissertation Submitted to the Senate of
Universiti Pertanian Malaysia in Fulfilment of the Requirements
for the Degree of Doctor of Philosophy.

PLANT REGENERATION AND BIOCHEMICAL CHANGES IN RICE (*Oryza sativa* L.) UNDER NaCl STRESS

BY

MOHAMMAD SAEED KHAN KHATTAK

October, 1996

Chairman **:Professor Hajjah Marziah Mahmood, Ph.D.**

Faculty :Science and Environmental Studies.

This project was designed to examine the effects of NaCl on growth and regeneration of callus and cell suspension cultures of the five rice cultivars namely, Puteh Perak, Mahsuri, Basmati-370, Nona Bokra and Khari Gunja. In addition, changes of nitrogen assimilating enzymes, phosphatase and some dehydrogenase of Kreb's Cycle in cell suspension cultures of Basmati-370 and Puteh Perak were also investigated. The callus cultures were induced from embryo section of seeds of rice using MS solid media containing 10 μM 2,4-D and 2 μM kinetin. Cell suspension cultures were initiated in liquid media from the induced embryogenic callus of rice using salts of the B5 media containing 10 μM 2,4-D. The five rice cultivars tested had high rates of callus induction (80-88%).

In the one step NaCl treatment, the growth of control callus and cell suspension cultures of the five rice cultivars decreased and significant morphological changes were observed. At 180 mM NaCl treatment , the reduction in callus growth was 29, 36, 42, 32 and 42% while at 70 mM NaCl, reduction in the growth of cell suspension culture was 38, 53, 58, 41 and 55% in Puteh Perak, Mahsuri, Basmati-370, Nona Bokra and Khari Gunja, respectively compared to the control culture without NaCl (100%). The callus growth was completely retarded at 540 mM NaCl treatment while in cell suspension it was at 210 mM NaCl. The multiple step NaCl-treated callus and cell suspension cultures of the five rice cultivars produced higher growth (% dry weight) in the presence of NaCl compared to the control cultures. The callus and cell

suspension of the five rice cultivars on high NaCl containing media reached a maximum growth at range of 10-13th passages and 6-9th passages, respectively while the control cultures proliferated at constant rate. The multiple step NaCl-treated cell suspension of Basmati-370 showed stability to NaCl treatment compared to the control.

MS solid regeneration media containing 3 μM IAA and 40 μM kinetin was most suitable for plant regeneration of callus and cell suspension of five rice cultivars. The plant regeneration capacity of one step NaCl-treated callus and cell suspension cultures of the five rice cultivars decreased with the increased concentrations of NaCl in the treatment media. Higher regeneration frequencies were observed from multiple step NaCl-treated callus of Puteh Perak, Mahsuri and Nona Bokra and cell suspension cultures of Puteh Perak and Nona Bokra compared to the control while lower regeneration than the control was obtained in other cultivars. The plant regeneration capacity of multiple step NaCl-treated and control cultures decreased with the increase in culture age. However, NaCl-treated cultures maintained higher regeneration capacity for up to 48 weeks of culture compared to the control.

The growth rate of cell suspension of Basmati-370 increased from day 4 up to day 10 and the activities of ACP, ALP, GS, MDH, NADP-ICDH and SDH were highest on day 10 while GDH and GOGAT on day 8 and NR on day 6 during the 14 days of culture period. The activities of GDH, GOGAT, NR, ACP, ALP, MDH and NADP-ICDH extracted from NaCl-treated cell suspension of Basmati-370 increased while GS and SDH activities decreased. The activities of GDH, NR, ALP and MDH decreased in cell suspension of Puteh Perak but increased in Basmati-370 under increasing NaCl treatments. The GS and SDH activities increased in the presence of NaCl of Puteh Perak but decreased in Basmati-370 and the GOGAT, ACP and NADP-ICDH increased by NaCl in both cultivars tested. The addition of NaCl (0-200 μM) to the assay mixture resulted in increased specific activities of GDH, NR, GOGAT, ACP, MDH and NADP-ICDH at low concentration but decreased the activities at high concentrations while the specific activities of GS, ALP and SDH continously decreased. Changes in selected enzyme activities of cell suspension of rice due to NaCl indicates that NaCl interferes with nitrogen and phosphorus metabolism.

Abstrak Dissertasi yang dikemukakan kepada Senat Universiti Pertanian Malaysia Sebagai Memuenuhi Syarat Keperluan Untuk Ijazah Doktor Falsafah

REGENERASI DAN PERUBAHAN BIOKIMIA POKOK PADI (*Oryza sativa* L.) DI BAWAH TEGASAN GARAM (NaCl)

Oleh

MOHAMMAD SAEED KHAN KHATTAK

October, 1996

Pengerusi :**Professor Hajjah Marziah Mahmood, Ph.D.**

Fakulti :Sains dan pengajian

Projek ini telah dijalankan untuk menentukan kesan NaCl ke atas pertumbuhan dan regenerasi kalus dan sel ampaian terhadap lima kultivar padi iaitu Puteh Perak, Mahsuri, Basmati-370, Nona Bokra dan Khari Gunja. Selain dari itu perubahan enzim yang mengasimilasikan nitrogen, fosfatase dan beberapa dehidrogenase pada kitaran Kreb's juga diuji. Kultur kalus diaruh dari embrio biji padi dengan menggunakan media pepejal MS yang mengandungi 10 μM 2,4-D dan 2 μM kinetin. Pengaruhan sel ampaian pula dilakukan pada kalus embriogenik dalam media cair B5 yang mengandungi 10 μM 2,4-D. Semua kultivar padi yang diuji memberikan kadar yang tinggi (80-88%) dalam pengaruhan kalus.

Perlakuan NaCl mengurangkan tumbesaran kalus dan sel ampaian serta mengubah ciri morfologinya bagi semua kultivar padi. Dengan pemberian 180 mM NaCl, tumbesaran kalus berkurangan sebanyak 29, 36, 42, 32 dan 42%, manakala dengan pemberian 70 mM NaCl, tumbesaran sel ampaian berkurangan sebanyak 38, 53, 58, 41 dan 55% berbanding dengan perlakuan tanpa NaCl masing-masing bagi kultivar Puteh Perak, Mahsuri, Basmati-370, Nona Bokra dan Khari Gunja. Kalus akan mati pada tahap rawatan 540 mM NaCl, manakala sel ampaian pada kepekatan 210 mM. Namun begitu secara amnya, kalus dan sel ampaian bagi kelima-

lima kultivar padi yang diperlakukan dengan NaCl memberikan tumbesaran yang lebih tinggi berbanding dengan kawalan. Kadar tumbesaran ini meningkat secara optimum pada kalus (10-13 'passages') dan sel ampaian (6-9 'passages') berbanding dengan kawalan yang memberikan kadar proliferasi yang lebih tetap. Sel ampaian dari kultivar Basmati-370 yang diperlakukan dengan NaCl adalah lebih stabil tumbesarannya apabila diberikan perlakuan NaCl.

Media pepejal MS yang mengandungi 3 μM IAA dan 40 μM kinetin adalah media yang paling sesuai untuk regenerasi kalus dan sel ampaian bagi semua kultivar padi. Kapasiti regenerasi dari kalus dan sel ampaian yang dirawat dengan NaCl berkurangan dengan peningkatan kepekatan rawatan NaCl dalam media. Frekuensi tertinggi regenerasi dari kultur kalus didapati pada kultivar Puteh Perak, Mahsuri, dan Nona Bokra, manakala pada sel ampaian ialah pada kultivar Puteh Perak dan Nona Bokra. Frekuensi regenerasi berkurangan dengan peningkatan umur kultur. Bagi kultur yang dirawat dengan NaCl memberikan regenerasi secara optimum pada umur 48 minggu.

Kadar pertumbuhan sel ampaian kultivar Basmati-370 meningkat pada hari ke-4 hingga hari ke-10. Kajian aktiviti enzim mendapati ACP, ALP, GS, MDH, NADP-ICDH dan SDH tertinggi pada hari ke-10, manakala aktiviti GDH dan GOGAT pada hari ke-8 dan NR pada hari ke-6. Aktiviti enzim GDH, GOGAT, NR, ACP, ALP, MDH dan NADP-ICDH dari sel ampaian kultivar Basmati-370 meningkat manakala aktiviti GS dan SDH menurun. Aktiviti GDH, NR, ALP dan MDH berkurangan pada sel ampaian kultivar Puteh Perak tetapi meningkat pada kultivar Basmati-370 dengan rawatan NaCl. Aktiviti GS dan SDH meningkat dengan kehadiran NaCl pada kultivar Puteh Perak, tetepi menurun pada Basmati-370. Aktiviti GOGAT, ACP dan NADP-ICDH meningkat dengan rawatan NaCl bagi bebrapa kultivar yang diuji. Penambahan NaCl dari 0-200 μM meningkatan aktiviti GDH, NR, GOGAT, ACP, MDH dan NADP-ICDH pada kepekatan yang rendah dan berkurangan pada kepekatan yang tinggi, tetapi aktiviti GS, ALP dan SDH berkurangan dengan peningkatan penambahan NaCl. Perubahan aktiviti enzim pada sel ampaian ini menunjukkan kehadiran NaCl menjejaskan metabolisme nitrogen dan fosforus.

CHAPTER 1
INTRODUCTION

Salinity is one of the most important constraints to crop production in the world. Of the fourteen billion hectares of available land in the world, only one fourth of it is potentially arable (Flowers et al. 1977). Nearly 25% of this arable land is subjected to salinity.

Rice (*Oryza sativa* L.) is the staple food for over half of the world population. There are only two cultivated species namely, *Oryza glaberrima* Steud. *and Oryza sativa* Linn. The cultivated species of *Oryza sativa* are classified into three subspecies, *Indica*, *Japonica* and *Javanica*. Rice is sensitive to salinity at whole plant level (Yeo et al. 1991). Toxic levels of salt limit the cultivation of rice on more than 50 million hectares of arid land or coastal plains of South and Southeast Asia. To develop salt-tolerant cultivars, it is necessary to understand the genetics of tolerance and the interactions between salinity and environmental factors. Genetic variability exists in salt tolerance, and is being used in breeding programmes (Moelijopawiro and Ikehashi, 1981). Tissue culture offers opportunities for generating variability (Lorz et al. 1988). *In vitro* techniques have been recognized as a promising tool for somaclonal variation to use in crop improvement and apply cellular selection for screening useful variants (Larkin and Scowcroft, 1981). The information available are scanty, however, the variation for a wide range of characteristics such as salinity tolerance, aluminium tolerance, disease tolerance, chlorophyll deficiency and morphological characters have been recorded (Vajrabhya et al. 1989; Daub, 1986; Conner and Meredith, 1985b; Suenaga et al. 1982).

The application of tissue culture techniques to the improvement of plant adaptation to environmental stress such as salt is relatively recent. Sodium chloride-tolerant cell lines of tobacco, sunflower, mung bean, sweet pepper, potato, bulrush millet and rice were obtained by exposing callus and cell suspensions to increasing

and different levels of NaCl (He and Yu, 1995; Gulati and Jaiwal, 1994; Prakas et al. 1993; Bhattacharya, 1991; Sabbah and Tal, 1990; Flowers et al. 1985; Rangan and Vasil, 1983, Croughan et al. 1981; Rains et al. 1980; Hasegawa et al. 1980; Nabors et al. 1975; Dix and Street, 1975).

The genetic variation of the somaclones recovered in regenerated plants has been considered to be a novel source for selection of agriculturally important traits (Evans and Sharpe, 1983). Nabors et al. (1980) were the first to regenerate plants from callus of tobacco tolerant to NaCl and the tolerance was transmitted to two additional generations. Salt tolerant lines of cells have also been selected from some other crops (Winicov, 1991; Croughan et al. 1981). In rice, reports are available on plant regeneration from callus and cell suspension cultures (Boissot et al. 1990; Zimny and Lorz, 1986; Abe and Futsuhara, 1986a & b, 1985, 1984; Chen et al. 1985; Cho and Zapata, 1988; Heyser et al. 1983; Jenes and Pauk, 1989; Kavi Kishor and Reddy, 1987) but very few reports are available on plant regeneration from callus and cell suspension cultures under salt stress (Niluffer and Zapata, 1994; Binh et al. 1992; Vajrabhaya et al. 1989; Woo et al. 1985; Wong et al. 1983).

Salt tolerance of plants is a complex phenomenon that involves development and morphological processes. These types of developmental and morphological changes have been periodically discussed in several reviews (Greenway and Munns, 1980; Flowers et al. 1977). Morphological and physiological changes in plants have been correlated with different metabolic abnormalities such as protein synthesis, enzyme changes, amino acid accumulation, carbohydrate and lipid accumulation (Dubey and Sharma, 1989; Basu et al. 1988).

Many researchers investigated the effects of salt (NaCl) on the metabolic activities of the major enzymes of different pathways in whole plants. (Dubey and Rani, 1990; Basu et al. 1988; Dubey et al. 1987; Pan, 1987; Sharma and Garg, 1985), however, very few researchers have exploited the tissue culture techniques for this type of study (Blits and Galgher, 1990; Larosa et al. 1989). Similarly, the effects of salt (NaCl) on the activities of the various enzymes in rice have been reported on whole

plant level (Dubey and Rani, 1990; Dubey and Sharma, 1989) but limited study on tissue culture system (Kavi Kishor, 1989; Flowers et al. 1985) was carried out.

In this study five cultivars of rice, two from Malaysia, namely, Puteh Perak and Mahsuri, and three from Pakistan, namely, Basmati-370, Nona Bokra and Khari Gunja, have been selected to study the effects of salt (NaCl) on various parameters using tissue culture system. The objectives of this study were:

1. To study the responses of callus and cell suspension cultures of rice to NaCl treatments.

2. To determine the plant regeneration capacity and stability of callus and cell suspension cultures of rice treated with NaCl.

3. To investigate the effects of salt (NaCl) on the activities of nitrogen assimilating enzymes, phosphatase and selected dehydrogenase in cell suspension cultures of rice.

CHAPTER 2
LITERATURE REVIEW

Importance of Tissue Culture

The application and utilization of tissue and cell culture techniques to the improvement of adaptation of plants to environmental stresses such as salts, acidity and drought are relatively new. The published work in this field especially on its genetic application is limited (Tal, 1983). Dix (1980), has briefly described the selection of cell lines to various environmental stresses. The use of plant tissue culture for mutant isolation was first reported in 1959 by Melchers and Bergmann. They described the selection for tolerance to temperature extremes in *Antirrhinum* suspension cultures. They have clearly described the use of cell culture for mutant selection, including the possibility of mass selection, screening of haploid cells and also regeneration of plants from selected cell lines. Subsequently, there was a series of similar reports by Carlson (1970), Heimer and Filner (1970) and Binding et al. (1974).

Mutant selection in cultured plant cells has been reviewed in great detail by Meliga (1980) and Chaleff (1981). Specific problems including the source and nature of heritable variation in culture, the use of tissue culture induced variability for crop improvement (Larkin and Scowcroft, 1981) and protoplast cultures in mutant selection (Bourgin, 1983 and Maliga, 1983) and the isolation of agronomically useful mutants from plant cell cultures (Chaleff, 1983) has since been reviewed.

Crop production is limited by high soil salt contents, water stress, and low or high temperature extremes (Nabors et al. 1980; Handa et al. 1982). Akbar and Ponnamperuma (1982) reported that there is 380 million hectares of saline soil on the surface of our earth. Out of this, 54 million hectares of strongly saline soil is in the South and Southeast Asia. In addition, the need of the land for irrigation has increased the problem of high salt concentration in the soil. Alternatively, various programmes