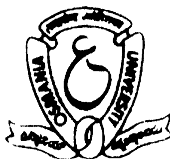
A study on the mechanisms of salinity tolerance and development of molecular markers in pearl millet [*Pennisetum glaucum* (L.) R. Br.]

Thesis Submitted to
Osmania University, Hyderabad

For The Award of the Degree of
Doctor of Philosophy in Genetics

By

Rupasree Mukhopadhyay
M.Sc. (Marine Biotech.)



DEPARTMENT OF GENETICS
OSMANIA UNIVERSITY
HYDERABAD 500 007
ANDHRA PRADESH, INDIA

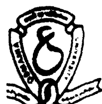
2006

Jaswant S. Kanwar Library
ICRISAT

To
My Family
Whose support and love has made it possible

And to
My Teachers
Who provided invaluable source of strength and inspiration

Department of Genetics
Osmania University
Hyderabad - 500 007, India
Phone (Off) 91-40-2768-2335
91-40-2709-5178
Res : 91-40-2766-0149
Fax : 91-40-2709-5178



Prof. P.B. Kavi Kishor
Professor and Co-ordinator, UGC-SAP (DSA)

Email : pbkavi@yahoo.com

CERTIFICATE

This is to certify that the thesis entitled **"A study on the mechanisms of salinity tolerance and development of molecular markers in pearl millet [*Pennisetum glaucum* (L.) R. Br.]"** in partial fulfillment of the requirement for the award of degree of Doctor of Philosophy in Genetics, Osmania University, Hyderabad, embodies the results of bonafide research work carried out by Ms. Rupasree Mukhopadhyay, under my supervision and guidance and that no part of the thesis has so far been submitted any where for any other degree or diploma.

The assistance and help taken during the course of the investigation and the source of literature and material has been duly acknowledged by her.

Date: 15-9-2006

Place: Hyderabad

(P.B. Kavi Kishor)

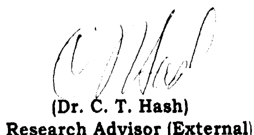
Research Supervisor

International Crops Research Institute for the Semi-Arid Tropics

Headquarters: Patancheru 502 324, Andhra Pradesh, India
Tel +91 40 30713071 **Fax** +91 40 30713074 +91 40 30713075 **Email** icrisat@cgiar.org
Liaison Office: CG Centers Block, NASC Complex, Dev Prakash Shastri Marg, New Delhi 110 012, India
Tel +91 11 25849552/25849553/25849554 **Fax** +91 11 25841294

CERTIFICATE

This is to certify that a part of the thesis entitled **"A study on the mechanisms of salinity tolerance and development of molecular markers in pearl millet [*Pennisetum glaucum* (L.) R. Br.]"** in partial fulfillment of the requirement for the award of degree of Doctor of Philosophy in Genetics, Osmania University, Hyderabad, was carried out by Ms. Rupasree Mukhopadhyay, under my advisory supervision and that it has not previously formed the basis for the award of any degree or diploma or other similar title.



Date: 8 Sep '06
Place: Patancheru, A.P.

(Dr. C. T. Hash)
Research Advisor (External)

ICRISAT-Bamako
BP 320
Bamako
Mali
Tel +223 2223375
Fax +223 2228683
Email icrisat-w-mali@cgiar.org

ICRISAT-Bulawayo
Matopos Research Station
PO Box 776,
Bulawayo, Zimbabwe
Tel +263 83 8311 to15
Fax +263 83 8253/8307
Email icrisatzw@cgiar.org

ICRISAT-Lilongwe
Chitedze Agricultural Research Station
PO Box 1096
Lilongwe, Malawi
Tel +265 1 707297/071/067/057
Fax +265 1 707298
Email icrisat-malawi@cgiar.org

ICRISAT-Maputo
c/o INIA, Av. das FPLM No 2698
Caixa Postal 1906
Maputo, Mozambique
Tel +258 1 461657
Fax +258 1 461581
Email icrisatmoz@panintra.com

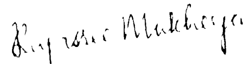
ICRISAT-Nairobi
(*Regional hub ESA*)
PO Box 39063, Nairobi,
Kenya
Tel +254 20 7224550
Fax +254 20 7224001
Email icrisat-nairobi@cgiar.org

ICRISAT-Niamey
(*Regional hub WCA*)
BP 12404
Niamey, Niger (Via Paris)
Tel +227 722529, 7227
Fax +227 734329
Email icrisatsc@cgiar.org

Visit us at www.icrisat.org

DECLARATION

I hereby declare that the research work entitled **"A study on the mechanisms of salinity tolerance and development of molecular markers in pearl millet [*Pennisetum glaucum* (L.) R. Br.]"** has been carried out by me in the Department of Genetics, Osmania University, Hyderabad-500 007, under the supervision of **Prof. P.B. Kavi Kishor**. The work done is original and no part of the thesis has been submitted for any other degree or diploma of any other University.



Date: 18 .4. 2 06

Place: Hyderabad

(Rupasree Mukhopadhyay)
M.Sc. (Marine Biotech.)
CSIR-SRF, Department of Genetics
Osmania University
Hyderabad -500 007

OUTLINE

CHAPTER	TITLE	PAGES
	Acknowledgements	**ii-iv**
	Preface	**v**
	Table of Contents	**vi-ix**
	List of Tables	**x-xi**
	List of Figures	**xii-xiii**
	List of Plates	**xiv-xv**
	List of Abbreviations and Symbols	**xvi-xviii**
Chapter 1	**Introduction**	**1-8**
Chapter 2	**Review of Literature**	**9-40**
Chapter 3	**Materials and Methods**	**41-62**
Chapter 4	**Results**	**63-82**
Chapter 5	**Discussion**	**83-112**
Chapter 6	**Summary and Conclusions**	**113-121**
Chapter 7	**Literature Cited**	**122-170**
Chapter 8	**Publications**	**171**
Chapter 9	**Appendices**	**172-187**

ACKNOWLEDGEMENTS

This is undoubtedly the pleasantest part of writing a thesis. Acknowledging the important roles played by other people in any activity is not only necessary, but also enjoyable. So here goes.

First and foremost, on this day I stand obliged and indebted to my Ph.D guide, ***Prof. P.B. Kavi Kishor****, Head, Department of Genetics, Osmania University Hyderabad. I owe my great devotion, respect and profound gratitude towards him for his unfailing enthusiasm, encouragement and generous help. He was always available for consultation and always gave me a patient hearing, even when he was extremely busy. His guidance and creative criticism enriched the quality of scientific component. He had been extremely understanding about my problems and shortcomings. I must also thank him for his insistence on maintaining high standards in all things.*

I wish to express my sincere, wholehearted gratitude to ***Dr. C. T. Hash****, Principal Scientist (Cereal Molecular Breeding), ICRISAT, Patancheru for giving me the opportunity to work at ICRISAT and for providing excellent research facilities with conducive environment He has been much more than an advisor; his constant encouragement enabled me to learn and grow throughout my research work. His insights into difficult problems were profound and original. It was purely his influence and help that enabled me to venture out a new marker technique in my work. I shall always remember his insistence that we could perform research that was truly second to none in the world. Indeed, I am honoured to be one of his students.*

I highly acknowledge the sponsored ***Junior*** *and* ***Senior Research Fellowships*** *by* ***CSIR, New Delhi****. I thank them for providing me the financial support in the form of fellowship without which this research work would not have been accomplished.*

I express deep sense of gratitude and sincere thanks to ***Dr. Dave Hoisington****, Global Theme Leader, Biotechnology, ICRISAT, Patancheru, for permitting me and providing the research facilities in the M. S. Swaminathan Applied Genomics Laboratory (AGL) and also for his fruitful suggestions in my work.*

It is my immense pleasure to express my utmost gratitude and reverence to ***Dr. S. Senthilvel****, Special Project Scientist, ICRISAT. His advices, patience, support and encouragement have no doubt enabled me to overcome all the hurdles in my molecular work. He was associated with my work since its initiation and was always available for valuable suggestions and corrections in my work. His vision and adept discussion technically and otherwise helped me to learn and understand the challenges of this field and the potential it has. I take the pride in having been associated with such a teacher.*

With pleasure, I thank ***Dr. Rajeev. K. Varshney****, who has been a great source of inspiration to me ever since I met him at AGL, ICRISAT. Although he got involved at a later stage in my research work, his suggestions and encouragement mean a lot to me and for my future endeavours.*

Thanks are also due to **Dr. Jayasree Balaji**, Bioinformatics Unit, ICRISAT, Patancheru for helping me with the primer-designing and her valuable suggestions.

I wish to thank **Dr. J. V. Rao**, Senior Scientist, Toxicology Unit, IICT, Hyderabad and his group, for helping me out with the antioxidative enzyme assays, which has added useful content to my research work.

My personal thanks to **Mr. Ramji** for not only helping me with my work at even the oddest times, but also for helping me in my CSIR- fellowship related matters. Without his help I could not have submitted my thesis. Thanks are due to **Mr. Mahesh** and **Mr. Chakra Reddy** for their help.

My special heartfelt thanks are due to **Dr. K.C. Majumdar**, **Dr. M.K. Chattopadhyay and their groups**, Center for Cellular and Molecular Biology, Hyderabad, for their intellectual discussion and kind help in allowing me to perform my protein-profiling experiments in their labs at the most difficult and crucial time of my research and life.

Grateful thanks are also due to all the **faculty members** of the **Department of Genetics**, Osmania University, Hyderabad for their help, encouragement and support.

I express my profound gratitude to **Mr. P. Ramu**, Scientific consultant, ICRISAT, for the help rendered to me in designing the primers and skillfully teaching me the invaluable bioinformatics tools. I do not have words to express my gratitude towards **Mr. R. Rajaram** and **Mr. C. Ashok Kumar** who were always available to solve any of my problems, apart from helping me in my work They have not only been very good friends in all respects but also a constant source of inspiration and encouragement throughout my work schedule at AGL, ICRISAT. I also express my sincere thanks to **Dr. Nepoleon**, Special Project Scientist, ICRISAT, for the help rendered to me while preparing the QTL maps. My heartfelt thanks are due to **Dr. Santosh P. Deshpande** for taking all the pains of teaching me in detail about QTL mapping and readily helping me out of the way for my research work, presentations and thesis writing. Special thanks are also due to **Dr. Ranjana Bhattacharjee** and **Dr. P. Lava Kumar** for their help and valuable suggestions, which helped me a lot during my thesis writing.

I would like to acknowledge with high spirits, the technical help rendered by **Mr. P. Surender Reddy** and **Ms. N. Jalaja** during the finishing stages of my research work at the Department of Genetics, Osmania University, Hyderabad. A lot of thanks go out to them.

I am grateful to **Mrs. Seetha Kannan**, Lab Manager, AGL, ICRISAT, **Mr. Bryan Moss**, Senior Research Technician and members of the Central Support Lab for their encouragement and co-operation during my lab work; other AGL Senior Research technicians mainly **Mr. Narsi Reddy**, **Mr. Gaffoor**, **Mr. Somaraju** and **Mr. Eshwar** for their invaluable help in teaching me certain lab skills which have helped a great deal in completing my work successfully.

To all the people in the **Department of Genetics, Osmania University** and **ICRISAT, Patancheru** where I spent most of my research life these past three years, heartfelt thanks for standing by my side in all ups and downs of my research. They made the lab more than just a workplace. In no particular order, many thanks to my friends: **Mrs. R. Naga Amrutha, Dr. G. Jogeswar, Dr. K. Rajender Rao, Ms. M.S.L.**

Sunitha*, *Mrs. Srilaxmi*, *Mrs. Jayasree*, *Mr. Ranadheer Kumar*, *Mr. Prashant*, *Ms. Sireesha* , *Ms. Durga V.*, *Mrs. D. Madhavi* , *Mr. R. Prasad*, *Mr. Nataraj Shekhar*, *Ms. Ranjana*, *Ms. Rohini*, *Ms. Swapna*, *Dr. Amita *at the Dept. of Genetics, O.U. Hyderabad;*, ***Dr. Mehtre Dr. S.K. Gulia*, *Mrs. N. Sridevi*, *Ms. Jyothi*, *Mrs. Prathibha*, *Ms. Anuradha Mr. Sripathi*, *Mr. Kassahaun*, *Mr. Velu G.*, *Mr. Bhushan K.*** *at ICRISAT, Patancheru.*

The aforementioned ***Mr. A. Krishna Reddy*****,** ***Mr. Bhaskar*****,** ***Ms. Shobha*****,** ***Mr. Ramdas*****,** ***Mr. Malla Reddy*** *and* ***Ms. Bakkamma*** *have done so much for me, way above and beyond the call of duty, that I must also acknowledge them separately. To all the Lab Assistants who have helped me through my research work often doing much more than their normal duties, special thanks are due from me.*

Special thanks are due to ***Mrs. S. Devi*** *of the Pearl Millet Breeding Office, ICRISAT for coming forward to help me whenever I approached her. Special thanks are also extended to* ***Library staff*****,** ***Pearl Millet Breeding Unit*** *staff, and* ***Learning Systems Unit*** *staff, especially,* ***Mr. S. V. Prasad Rao*****,** *for their excellent assistance during my research work at ICRISAT. Thanks are due to* ***Mr. N. Chary*** *for the wonderful photographs of my research work.*

The completion of my doctoral research would not have been possible without the company, cooperation and good will of many of my close ***friends*** *and* ***labmates****. I thank all my friends for sharing every emotion of mine and keeping me fit and fine through out. I convey my wholehearted thanks to all my well wishers and other friends requesting their forgiveness for not mentioning them here by name.*

Any appreciable word would be less to thank my ***mother*** *and* ***mother-in-law*** *for the blessings they showered on me. My career would not have progressed in this direction and to this level without their generous help, understanding and encouragement. Their love and affection brought me to the level where I am today and also kindled my enthusiasm to carry out my research.*

I cherish all the pleasant moments with my brother, ***Rajarshi*** *and sister-in-law,* ***Rachna****, who, apart from their support and encouragement, have filled in very valuable and helpful suggestions, which fetched me a lot of appreciation during my presentations and thesis writing.*

Last but not the least, much love and thanks to my family, especially my ***husband*** *and my* ***son*****,** *whom I have neglected a lot in due course of my research work. Without their understanding, encouragement and co-operation, this research work would not have been possible at all.*

Rupasree Mukhopadhyay

PREFACE

Pearl millet, [*Pennisetum glaucum* (L.) R. Br.], the sixth most important cereal crop with 2n=2x=14, is grown predominantly in Africa and Asia, as a staple food grain and source of feed, fodder, fuel and construction material in the hottest, driest, semi arid and arid regions. In recent times, there is a renewed interest globally in growing pearl millet because of its drought tolerance and high quality grain. Despite its importance, pearl millet can be considered an "orphan" crop because of its vast untapped potential, less sequenced genome and limited markers for genetic studies. Additionally, pearl millet productivity is hampered to a great extent by soil salinity. Thus, research for increasing the salt tolerance of pearl millet will not only increase the productivity of this important crop, but also allow the more effective use of poor quality irrigation water in salt affected areas.

"A study on the mechanisms of salinity tolerance and development of molecular markers in pearl millet [Pennisetum glaucum (L.) R. Br.]" is aimed mainly to assess opportunities for using existing pearl millet populations (Hash and Witcombe, 1994; Hash et al., 2001) and other pearl millet genetic stocks available at ICRISAT-Patancheru to generate molecular markers for genomic regions contributing to salinity stress tolerance during germination and early seedling growth. Salinity tolerance during these early growth stages is critical to crop establishment in saline soil conditions and soil free *in vitro* screens can be used as a way of reducing the complexity of genotype × environment interactions to assess this on the large numbers of entries required for phenotyping a mapping population progeny set.

This thesis is broadly divided into 9 chapters. Chapters 1 and 2 contain the introduction and review of literature, respectively; chapter 3 deals with materials and methods used; chapter 4 represents the experimental results. Chapter 5 contains the discussion of the results. The overall summary and conclusions are presented in chapter 6, and the cited literature in the chapter 7. Chapter 8 contains the list of publications and presentation of papers in symposia and conferences in due course of research work. The details of the composition of buffers and reagents and score-sheets of marker data analysis are included in chapter 9.

TABLE OF CONTENTS

1 Introduction

2 Review of Literature
2.1 Salinity -a major problem hampering crop productivity
2.2 Types and Causes of Salinity
2.2.1 Natural or primary salinity
2.2.2 Secondary salinization or human-induced salinity
2.3 The Effect of Salinity on Plants
2.4 Salt Tolerance
2.4.1 Influence of growth stage on salinity tolerance
2.5 Mechanisms of salt stress tolerance
2.5.1 Selective accumulation or exclusion of ions
2.5.2 Synthesis of compatible solutes
2.5.3 Ion homoeostasis or control of ion uptake
2.5.4 Induction of antioxidative enzymes by salinity
2.6 Pearl millet: current status and future potential
2.6.1 Uses of pearl millet
2.6.2 Nutritive value of pearl millet
2.6.2.1 Food Value
2.6.2.2 Feed Value
2.6.3 Importance of pearl millet for research
2.6.3.1 Breeding
2.6.3.2 Molecular mapping in pearl millet
2.6.4 Pearl millet and salinity tolerance
2.7 Molecular marker analysis
2.7.1 DNA markers
2.7.1.1 Target Region Amplification Polymorphism (TRAP)
2.7.2 Linkage mapping
2.7.2.1 Linkage maps in pearl millet
2.7.3 Marker-trait associations
2.8 QTL mapping
2.8.1 Methods to detect QTLs
2.8.2 QTL mapping in pearl millet
2.8.3 QTL mapping for salinity tolerance

3 Materials and Methods
3.1 Seed Materials
3.2 Screening of Pearl Millet Inbred Lines for Salt Stress Tolerance
3.2.1 Materials
3.2.2 Surface sterilization and germination of seeds to early seedling stage
3.2.3 Morphological parameters
3.2.3.1 Germination efficiency (%)
3.2.3.2 Length of shoot and root (cm)
3.2.3.3 Root/shoot ratio
3.2.3.4 Fresh and dry weight (mg) of shoots and roots
3.3 Biochemical Parameters
3.3.1 Estimation of proline
3.3.2 Measurement of Na^+ and K^+ ions
3.3.3 Salt stress induced antioxidant responses

3.3.3.1 Preparation of enzyme extracts
3.3.3.2 Estimation of glutathione
3.3.3.3 Enzyme assays
3.3.3.4 Lipid peroxidation
3.3.3.5 Isoenzyme studies
3.3.3.5.1 Isoenzyme activity of superoxide dismutase (SOD)
3.3.3.5.2 Isoenzyme pattern of catalase
3.3.3.5.3 Isoenzyme activity of ascorbate peroxidase
3.3.4 Protein profiles
3.3.4.1 Protein extraction
3.3.4.2 Protein estimation
3.3.4.3 SDS-PAGE
3.3.5 Effect of GA_3 and $CaCl_2$
3.4 Statistics
3.5 Development of Molecular Markers for Salt Stress Tolerance
3.5.1 Plant material
3.5.2 Data mining for the DNA sequences of target genes
3.5.3 Primer design
3.5.3.1 Fixed / EST primers
3.5.3.2 Arbitrary primers
3.5.4 Genomic DNA extraction
3.5.4.1 DNA extraction and purification
3.5.4.2 Determination of quantity and quality of isolated DNA
3.5.5 PCR amplification
3.5.6 Testing parental polymorphism using TRAP markers
3.5.7 Poly-Acrylamide Gel Electrophoresis (PAGE)
3.5.7.1 Material
3.5.7.2 PAGE gel preparation
3.5.7.3 Silver staining
3.5.8 Marker data analysis
3.5.9 Construction of genetic linkage map
3.6 QTL analysis
3.6.1 Phenotypic data
3.6.2 Analysis of variance (ANOVA)
3.6.3 QTL mapping

4 Results
4.1 Screening and selection of pearl millet germplasm for salinity tolerance
4.1.1 Morphological parameters
4.1.2 Biochemical parameters
4.1.2.1 Proline estimation
4.1.2.2 Estimation of Na^+ and K^+ ions under salt stress
4.1.2.3 Effect of short-term salt stress on accumulation of proline.
4.1.2.4 Effect of short-term salt stress on accumulation of ions.
4.2 Antioxidative enzyme activity at short-term salt stress
4.2.1 Antioxidative enzyme activities of three representative lines of pearl millet
4.2. .1 Catalase (CAT)
4.2. .2 Superoxide dismutase (SOD)
4.2. .3 Glutathione reductase (GR)
4.2. .4 Glutathione-S-transferase (GST)
4.2. .5 Reduced Glutathione (GSH) content
4.2. .6 Lipid peroxidation
4.2.2 Antioxidative enzyme activities of pearl millet lines under continuous salt stress for 7 days at different salinity levels

4.2.2.1 Catalase (CAT)
4.2.2.2 Superoxide dismutase (SOD)
4.2.2.3 Glutathione reductase (GR)
4.2.2.4 Glutathione-S-transferase (GST)
4.2.2.5 Reduced glutathione (GSH) content
4.2.2.6 Lipid peroxidation
4.2.3 Antioxidative enzyme activities of parental lines of pearl millet with extreme responses to salt stress
4.2.3.1 Catalase (CAT)
4.2.3.2 Superoxide dismutase (SOD)
4.2.3.3 Glutathione reductase (GR)
4.2.3.4 Glutathione-S-transferase (GST)
4.2.3.5 Reduced Glutathione (GSH) content
4.2.3.6 Lipid Peroxidation
4.2.4 Isoenzyme profiles of five representative accessions of pearl millet
4.3 Protein profiles at short-term salt stress in pearl millet
4.3.1 Short-term salt stress at 0mM, 75mM & 150mM NaCl for 0h, 24h, 40h & 72h in four accessions of pearl millet
4.3.2 Protein content of seedlings at short-term salt stress
4.3.3 Short term salt stress of five accessions of pearl millet at 150mM NaCl for 0h, 24h, 48h, 72h, 96h, 120h, 144h and 168h
4.4 Effect of GA_3 and $CaCl_2$ on response to salinity stress in pearl millet seedlings
4.5 Development of molecular markers
4.5.1 Data mining and primer designing for TRAP markers
4.5.2 Optimization of TRAP technique
4.5.3 TRAP marker analysis
4.5.4 Construction of linkage map
4.5.5 QTL analysis
4.5.5.1 Phenotypic data analysis
4.5.5.2 QTL mapping

5 Discussions
5.1 Screening and selection of pearl millet germplasm for salinity tolerance
5.1.1 Morphological parameters
5.1.1.1 Effect of salt stress on germination
5.1.1.2 Effect of salt stress on length, fresh and dry weights of shoots and roots
5.1.2 Biochemical parameters
5.1.2.1 Proline accumulation
5.1.2.2 Accumulation of Na^+, K^+ and other ions under salt stress
5.2 Antioxidative enzyme activity
5.2.1 Antioxidative enzyme responses under short term salt stress
5.2.2 Isoenzyme profiles at short term salt stress
5.3 Protein profiles
5.4 Effect of GA_3 and $CaCl_2$ on salinity stress
5.5 Development of molecular markers
5.5.1 The advantage of TRAP markers
5.5.2 Optimization of TRAP technique for pearl millet
5.5.3 TRAP marker analysis
5.5.4 Construction of linkage map
5.5.4.1 Comparison with previously published maps
5.5.5 QTL analysis
5.5.5.1 Phenotypic data analysis
5.5.5.2 QTL mapping

6 Summary and Conclusions
6.1 Screening for salinity stress and possible mechanisms of salt tolerance in pearl millet
6.2 Development of molecular markers associated with salt tolerance in pearl millet

7 Literature Cited

8 Publications

9 Appendices
I GRAMENE BLAST search results and aligned sequences
II Composition of solutions and reagents
III Score-sheet for TRAP markers

LIST OF TABLES

Table No.	Title	Between Pages
2.1	Regional distribution of salt-affected soils, in million hectares	9-10
2.2	Molecular marker techniques	26-27
2.3	Advantages and disadvantages of most commonly used DNA markers for QTL analysis	26-27
3.1	Salient features of twenty-eight inbred lines of pearl millet tested for salt stress tolerance	41-42
3.2	List of fixed primers designed in this study	52-53
3.3	List of arbitrary primers designed in this study	53-54
4.1	ANOVA mean sums of squares for ten characters in 28 pearl millet inbred lines screened *in vitro* for seed germination (all 28 entries) and early seedling growth (germinating entries only) across three salt concentrations (C2-C4) and a non-saline control (C1)	64-65
4.2	ReML-adjusted entry means for 28 pearl millet inbreds screened *in vitro* in Hoagland's solution for germination in three treatments varying in NaCl concentration and in a non-saline control treatment	64-65
4.3	ReML-adjusted entry means for 28 pearl millet inbreds screened *in vitro* in Hoagland's solution for shoot and root lengths and root/shoot ratio (based on lengths) in three treatments varying in NaCl concentration and in a non-saline control treatment	64-65
4.4	ReML-adjusted entry means for 28 pearl millet inbreds screened *in vitro* in Hoagland's solution for shoot and root fresh weights and root/shoot ratios (based on fresh weights) in three treatments varying in NaCl concentration and in a non-saline control treatment	64-65
4.5	REML-adjusted entry means for 28 pearl millet inbreds screened *in vitro* in Hoagland's solution for shoot and root dry weights and root/shoot ratios (based on dry weights) in three treatments varying in NaCl concentrations and in a non-saline control treatment	64-65
4.6	Relative shoot and root length (%) of 10-day old seedlings of 27 pearl millet accessions germinated and grown at four NaCl concentrations	65-66
4.7	Relative shoot and root dry weight (%) of 10-day old seedlings of 27 pearl millet accessions germinated and grown at four NaCl concentrations	65-66

List of Tables contd.

Table No.	Title	Between Pages
4.8	Pearl millet lines categorized for response to salinity stress	66-67
4.9	Proline content (μg/mg dry wt. tissue) under short-term salt stress at 150mM NaCl from three groups of representative accessions of pearl millet seedlings	69-70
4.10	Ion content (mg/g dry wt.) from three groups of representative accessions of pearl millet seedlings under short-term salt stress at 150mM NaCl	69-70
4.11	Total Protein content (mg/g dry wt.) in pearl millet lines subjected to short-term salt stress at 150mM NaCl	76-77
4.12	Details of TRAP markers developed in this study	79-80
4.13	Chi-square analysis of segregation distortion of TRAP markers	79-80
4.14	Linkage group assignment and distribution of markers and length of linkage groups in the Tift $23D_2B_1$P1-P5 × WSIL-P8 based mapping population	80-81
4.15	ReML-adjusted entry means for (Tift $23D_2B_1$-P1-P5 × WSIL-P8)-based pearl millet mapping population screened *in vitro* in Hoagland's solution for germination, shoot length, root length and other derived traits in 150mM NaCl and in a non-saline control	81-82
4.16	Details of the putative QTLs identified associated with salinity tolerance traits using $F_{2:4}$ self bulk progenies of the pearl millet (Tift $23D_2B_1$-P1-P5 × WSIL-P8) based mapping population and CIM implemented in PlabQTL	82-83

LIST OF FIGURES

Figure No.	Title	Between Pages
2.1	Global distribution of salt-affected soils	9-10
2.2	Effect of saline soil on establishment and growth of a pearl millet hybrid in Rajasthan, India (2004)	24-25
3.1	96-well plate high throughput DNA extraction	56-57
3.2	Robotics for 96 well DNA dilutions	56-57
4.1	Changes in germination percentage of seeds of 28 pearl millet inbred lines across four salinity levels	63-64
4.2	Changes in shoot lengths of 28 pearl millet inbred lines germinated and grown at four salinity levels	64-65
4.3	Changes in root lengths of 28 pearl millet inbred lines germinated and grown at four salinity levels	64-65
4.4	Changes in shoot fresh weights of 28 pearl millet inbred lines germinated and grown at four salinity levels	64-65
4.5	Changes in root fresh weights of 28 pearl millet inbred lines germinated and grown at four salinity levels	64-65
4.6	Changes in shoot dry weights of 28 pearl millet inbred lines germinated and grown at four salinity levels	64-65
4.7	Changes in root dry weights of 28 pearl millet inbred lines germinated and grown at four salinity levels	64-65
4.8	Plots of responses between NaCl concentrations and root length (cm) of pearl millet seedlings from three groups of representative accessions	65-66
4.9	Proline content (µg/mg dry wt. tissue) under non-saline conditions and three levels of salt stress in 28 inbred lines of pearl millet	66-67
4.10	Plots of responses between NaCl concentrations and proline content (µg/mg dry wt. tissue) of pearl millet seedlings from three groups of representative accessions	67-68
4.11	(A) Na^+ content (% ash wt. basis), (B) K^+ content (% ash wt. basis) and (C) K^+/Na^+ ratios in salt treated seedlings of 27 pearl millet inbred lines as compared to their controls	68-69
4.12	Changes in antioxidative enzyme activity (A) Catalase, (B) Superoxide dismutase, (C) Glutathione reductase, (D) Glutathione S-transferase, (E) Reduced Glutathione content and (F) Lipid peroxidation during short-term salt stress at 150mM NaCl in three representative lines of pearl millet	71-72

List of Figures contd.

Figure No.	Title	Between Pages
4.13	Changes in antioxidative enzyme activity (A) Catalase, (B) Superoxide dismutase, (C) Glutathione reductase, (D) Glutathione-S-transferase, (E) Reduced Glutathione content and (F) Lipid peroxidation during salt stress for 7 days at different concentrations of NaCl in three representative lines of pearl millet	73-74
4.14	Changes in antioxidative enzyme activity (A) Catalase, (B) Superoxide dismutase, (C) Glutathione reductase, (D) Glutathione-S-transferase, (E) Reduced Glutathione content and (F) Lipid peroxidation during short term salt stress at 150mM NaCl in parental lines of pearl millet, WSIL P8 (salt sensitive) and Tift $23D_2B_1$-P1-P5 (highly salt tolerant)	74-75
4.15	Effect of $CaCl_2$ and GA_3 on the germination percentage of two inbred lines of pearl millet, ICMB 90111and 841B-P3 (sensitive and tolerant to salt stress, respectively) under 75mM, 100mM and 150mM NaCl stress as compared to their respective 0mM NaCl controls	77-78
4.16	Effect of $CaCl_2$ and GA_3 on the (a) shoot length and (b) root length of two inbred lines of pearl millet, ICMB 90111and 841B-P3, (sensitive and tolerant to salt stress, respectively) under 75mM, 100mM and 150mM NaCl stress as compared to their respective 0mM NaCl controls	77-78
4.17	Effect of $CaCl_2$ and GA_3 on the (a) shoot fresh weights and (b) root fresh weights, of two inbred lines of pearl millet, ICMB 90111and 841B-P3, (sensitive and tolerant to salt stress, respectively) under 75mM, 100mM and 150mM NaCl stress as compared to their respective 0mM NaCl controls	77-78
4.18	Effect of $CaCl_2$ and GA_3 on the proline content of two inbred lines of pearl millet, ICMB 90111 and 841B-P3, (sensitive and tolerant to salt stress respectively) under 75mM, 100mM and 150mM NaCl stress as compared to their respective 0mM NaCl controls	77-78
4.19	Genetic map of a pearl millet population that segregates for salt tolerance showing the distribution of molecular markers across the 7 linkage groups	80-81
4.20	Promising QTLs for shoot length and relative shoot length under saline conditions identified on LG6 of pearl millet	82-83

LIST OF PLATES

1	Ten-day-old seedlings of pearl millet lines grown in 0 mM, 75 mM, 100 mM and 150 mM NaCl media.	66-67
2	Isoenzyme patterns of superoxide dismutase in pearl millet lines (A) WSIL-P8, (B) ICMB 90111, (C) 863B-P2, (D) Tift $23D_2B_1$-P1-P5 and (E) 841B-P3 at 150mM NaCl in short-term salinity treatments	75-76
3	Protein profiles of 0mM, 75mM and 150mM NaCl treated seedlings of pearl millet for (A) 24 h, (B) 48 h and (C) 72 h treatments	76-77
4	Protein profiles of five accessions of pearl millet at 150mM NaCl for short-term salt stress for 0h (0), 24h (1), 48h (2), 72h (3), 96h (4), 120h (5), 144h (6) and 168h (7)	76-77
5	Effect of $CaCl_2$ (B), GA_3 (C) and both [GA_3+$CaCl_2$] (D) as compared to the control (A) in 10-day old seedlings of pearl millet line ICMB 90111 grown in 0mM, 75mM, 100mM and 150mM NaCl media	77-78
6	Effect of $CaCl_2$ (B), GA_3 (C) and both [GA_3+$CaCl_2$] (D) as compared to the control (A) in 10 day-old seedlings of pearl millet line 841B-P3 grown in 0mM, 75mM, 100mM and 150mM NaCl media	77-78
7	Optimization of PAGE gel profiles for detecting TRAP polymorphisms between Tift $23D_2B_1$-P1-P5 (parent A) and WSIL-P8 (parent B) with (a) P5CSF and (b) GR6F fixed TRAP primers in combination with arbitrary TRAP primers Ar1 to Ar15	78-79
8	Optimization of TRAP technique among the mapping population progenies of Tift $23D_2B_1$-P1-P5 × WSIL-P8 with TRAP primer combinations (A) GR6F-Ar2 and (B) SOD3F-Ar4	78-79
9	Glutathione reductase (GR) based TRAP polymorphisms in Tift $23D_2B_1$-P1-P5 (parent A) and WSIL-P8 (parent B)	79-80
10	Superoxide dismutase (SOD) based TRAP polymorphisms in Tift $23D_2B_1$-P1-P5 (parent A) and WSIL-P8 (parent B)	79-80
11	Δ^1-pyrroline-5-carboxylate synthetase (P5CS) based TRAP polymorphisms in Tift $23D_2B_1$-P1-P5 (parent A) and WSIL-P8 (parent B)	79-80
12	TRAP marker profiles seen among the mapping population progenies of Tift $23D_2B_1$-P1-P5 × WSIL-P8 with primer combination GR6F-Ar2	79-80

List of Plates contd.

13	TRAP marker profiles seen among the mapping population progenies of Tift $23D_2B_1$-P1-P5 × WSIL-P8 with primer combination GR8F-Ar4	79-80
14	TRAP marker profiles seen among the mapping population progenies of Tift $23D_2B_1$-P1-P5 × WSIL-P8 with primer combination SOD3F-Ar4	79-80
15	TRAP marker profiles seen among the mapping population progenies of Tift $23D_2B_1$-P1-P5 × WSIL-P8 with primer combination SOD3F-Ar11	79-80
16	TRAP marker profiles seen among the mapping population progenies of Tift $23D_2B_1$-P1-P5 × WSIL-P8 with primer combination P5CSF-Ar1	79-80
17	Screening for salinity stress related phenotypic traits for some randomly selected (Tift $23D_2B_1$ P1 P5 × WSIL-P8) $F_{2:4}$ mapping progenies in two salinity levels (0mM and 150mM NaCl)	81-82

LIST OF ABBREVIATIONS AND SYMBOLS

%	: Percent
°C	: Degree Celsius
/	: Per
μg	: Microgram
μl	: Microlitre
μM	: Micromolar
A	: Absorbance
AFLP	: Amplified Fragment Length Polymorphism
AP-PCR	: Arbitrary-Primed PCR
APS	: Ammonium persulphate
BLAST	: Basic Local Alignment Search Tool
BLUPs	: Best Linear Unbiased Predictions
bp	: Base pair
BSA	: Bovine serum albumin
CAPs	: Cleaved Amplified Polymorphic sequences
cDNA	: Complementary DNA
CDNB	: 1-chloro-2-dinitrobenzene
CIM	: Composite Interval Mapping
cM	: Centi Morgan
cm	: Centimeter(s)
CTAB	: Cetyl Trimethyl Ammonium Bromide
CV	: Co-efficient of Variance
Da	: Dalton
DFID	: Department For International Development
DH	: Doubled haploid
dH_2O	: Distilled water
DNA	: DeoxyriboNucleic Acid
dNTP	: Deoxyribose Nucleotide Tri-Phosphate
ds	: Double stranded
DTNB	: Dithionitrobenzoic acid
DTT	: Dithiothreitol
EDTA	: Ethylene Diamine Tetra Acetic acid
ESTs	: Expressed Sequence Tags
EtOH	: Ethanol
F	: Forward
fwt.	: Fresh weight
g	: Gram(s)
GR	: Glutathione reductase
GSA	: Glutamic-γ-semialdehyde
GSH	: Reduced glutathione
GSSG	: Oxidized glutathione
GST	: Glutathione-S-transferase
h	: Hour(s)
h^2	: Heritability
ha	: Hectare
HSPs	: High Scoring-segment Pairs
IAA	: Iso-amyl Alcohol
ICRISAT	: International Crops Research Institute for the Semi-Arid Tropics
ILRI	: International Livestock Research Institute
K	: Kilo
kb	: Kilo-base pair
kDa	: Kilo Daltons

kg	: Kilogram
L	: Litre(s)
LOD	: Likelihood odd ratio (Logarithm of Odds)
M	: Molar
m	: Metre
Mha	: Million hectares
MAS	: Marker Assisted Selection
max	: Maximum
Mbp	: Million base pairs
MDA	: Malondialdehyde
mg	: Milligram
min	: Minute(s)
ml	: Millilitre(s)
mM	: Millimolar
mm	: Millimeter(s)
mol. wt.	: Molecular weight
mRNA	: Messenger RNA
Mt	: Million tons
NAA	: α-Naphthaleneacetic acid
NaCl	: Sodium Chloride
NADPH	: Nicotinamide adenine dinucleotide phosphate (reduced)
NBT	: Nitroblue tetrazolium chloride
NCBI	: National Centre for Biotechnology Information
ng	: Nanogram
NIL	: Near Isogenic Lines
nm	: Nanometer
No.	: Number(s)
nt	: Nucleotide(s)
OD	: Optical Density
ORF	: Open Reading Frame
P5C	: Pyrroline-5-carboxylate
P5CDH	: Pyrroline-5-carboxylase dehydrogenase
P5CR	: Pyrroline-5-carboxylate reductase
P5CS	: Δ^1-Pyrroline-5-carboxylate synthetase
PAGE	: Poly Acrylamide Gel Electrophoresis
PCR	: Polymerase Chain Reaction
PDH	: Proline dehydrogenase
pH	: Hydrogen ion concentration
PMSF	: Phenyl methyl sulfonyl floride
QTL	: Quantitative Trait Loci
R	: Reverse
RAPD	: Random Amplified Polymorphic DNA
ReML	: Residual Maximum Likelihood
RFLP	: Restriction Fragment Length Polymorphism
RIL	: Recombinant Inbred Lines
Rnase	: Ribonuclease
rpm	: Revolutions per miniute
s	: Seconds
SCAR	: Sequence Characterized Amplified Region
SDS	: Sodium Dodecyl Sulphate
SE	: Standard Error
SIM	: Simple Interval Mapping;
SNP	: Single Nucleotide Polymorphism;
SOD	: Superoxide dismutase

SRAP	: Sequence Related Amplification Polymorphism
SSCP-SNP	: Single Stranded Conformational Polymorphism -Single Nucleotide Polymorphism
SSR	: Simple Sequence Repeats (microsatellites)
STS	: Sequence Tagged Site
TBE	: Tris Borate EDTA
TEMED	: N,N,N,N-tetra methylethylene diamine
Tm	: Temperature of melting
TRAP	: Target Region Amplification Polymorphism
U	: Unit(s)
UV	: Ultra violet
V	: Volt(s)
W	: Watt(s)
Δ	: Delta

INTRODUCTION

CHAPTER 1

INTRODUCTION

Salinity stress: one of the major environmental constraints to crop productivity globally

Saline soils are a problem for agriculture in many parts of the world, especially in arid and semi-arid regions where low precipitation, irrigation with brackish water and poor drainage interact to bring about soil salinity. Excess amounts of salt in the soil adversely affects plant growth and development leading to diminished economic yields (Bernstein, 1975) and poor quality of produce; limiting the productivity of crop plants (Ashraf, 1999). Approximately 20% of the world's cultivated area and nearly half of the world's irrigated lands are affected by salinity (Zhu, 2001a). Over 800 million hectares of land throughout the world are salt-affected, either by salinity (397 million ha) or the associated condition of sodicity (434 million ha) as reported by FAO (2005). In fact no continent on our planet is free from salt-affected soils. India for instance, has about 7 million ha of saline lands, of which 2 million ha occur in deltas in a strip ranging from a few kilometers to 50 km from the coast. The immense potential of salt-affected soils for much needed production of food, fiber, fuel, and forage crops is now more relevant than ever; production demands are increasing due to the growing population, and there is scant possibility of bringing new land under cultivation. This emphasizes the urgent need to increase productivity of salt-affected soils and help innumerable low-income small farmers to improve their lot.

The technology of combating salinity is extremely cost-extensive requiring large expenditures of energy to reclaim land and maintain soil balances. Also the ability of the crop to tolerate a given level of salinity becomes paramount in managing water and soil resources. An alternative to expensive large-scale irrigation and drainage schemes is the development of salt-tolerant crop species and for this reason there has been an upsurge of interest towards tailoring crop plants to suit more saline environments.

The majority of crop species are extremely susceptible to salt and most are unable to tolerate concentrations higher than 100 mol m^{-3} NaCl. Salt stress results in reduced water potential, ion imbalance and toxicity, which in turn lead to changes in development, growth and productivity and severe stress, may even threaten survival (Hasegawa *et al.*, 2000b). High concentrations of salts cause ionic, osmotic and associated secondary stresses to plants. Plant responses to these primary and secondary stresses are complex and can be grouped into three general categories: homoeostasis, detoxification and growth control (Zhu, 2001a). Homoeostatic responses include activities that help restore both ionic and osmotic balances in plant cells. Induction of metabolites and stress proteins that alleviate oxidative damage, and up-regulation of proteins that help to renature or remove denatured proteins that increase under stress are examples of detoxification. Growth control refers to the coordination of stress adaptation and the rate of cell division and expansion.

Salt effects are a combined result of the complex interactions among different morphological, physiological and biochemical processes. Morphological symptoms are indications of the injurious effects of salt stress. The extent of inhibitory or adverse effects can be known only by making critical comparisons with plants growing under comparable conditions in normal soils. Salinity may directly or indirectly inhibit cell division and enlargement in the plant's growing points. Reduced shoot growth caused by salinity originates in growing tissues and not in mature photosynthetic tissues (Munns *et al.*, 1982). As a result, leaves and stems of the affected plants appear stunted. Chloride induces elongation of the palisade cells, due to which the leaves become succulent. Salt stress hastens phenological development, *i.e.* induces early flowering in wheat (Francois *et al.*, 1986). It also reduces dry matter content, increases the root : shoot ratio, and diminishes leaf size in crop plants; as a result of which, grain yield is reduced. This grain yield reduction is attributed to reduced numbers of seeds, spikelets, and tillers, as well as low grain weight. Excess salt in the soil solution may adversely affect plant growth either through osmotic inhibition of water uptake by roots or by specific ion effects. The presence of high Na^+ and Cl^- concentrations and an altered water status in the soil brings about changes in plant metabolism, membrane disorganization, generation of reactive oxygen

species (ROS), metabolic toxicity, inhibition of photosynthesis and altered nutrient acquisition (Hasegawa *et al.*, 2000b). Specific ion effects may cause direct toxicity or, alternatively, the insolubility or competitive absorption of ions may affect the plant's nutritional balance. These effects may be associated with enzyme activity, hormonal imbalance, or morphological modifications. It should be noted that the relative roles of osmotic and specific ion phenomena in explaining the observed effects is disputed. Even at low salinity levels, external salt concentration is much greater than that of nutrient ions, so that a considerable concentration of ions may reach the xylem. Being the actively transpiring parts of the plant, the leaves accumulate salt to excessive levels, exceeding the ability of the cells to compartmentalize these ions in the vacuole (Munns and Termat, 1986). Ions then build up rapidly in the cytoplasm and inhibit enzyme activity or they build up in the cell walls and dehydrate the cell, which leads to their premature death (Flowers and Yeo, 1986; Munns and Passioura, 1984).

There are various mechanisms by which plants can protect themselves from abiotic stresses by accumulation of osmoprotectants, exclusion of ions, compartmentation of ions, transporter and symporter systems, water channels, chaperones, reactive oxygen species scavenging machinery and signaling molecules. Osmoregulation by accumulation of organic or inorganic solutes ensures that adequate turgor is maintained in the cell. The compounds that accumulate most commonly are proline and glycinebetaine, although other molecules can also accumulate to high concentrations in certain species (Hasegawa *et al.*, 2000b). Organic compounds that accumulate in the cytoplasm may function as osmotica and thereby protect the conformation of macromolecules in the changing ionic environment (Wyn Jones and Pollard, 1983). When plants are subjected to salt stress, the balance between the production of reactive oxygen species and the quenching activity of the antioxidants gets upset, often resulting in oxidative damage (Gosset *et al.*, 1994a). Plants possess a number of antioxidant enzymes that protect them from these potential cytotoxic effects due to the excessive generation of active oxygen species such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (Dionisio-Sese and Tobita, 1998; McCord, 2000; Hernandez *et*

al., 2001; Lee *et al.*, 2001). Superoxide dismutase (SOD) is a major scavenger of O_2^-, and its enzymatic action results in the formation of H_2O_2. Catalase and a variety of peroxidases (Chang *et al.*, 1984; Chen and Asada, 1989) catalyze the breakdown of H_2O_2. Thus, understanding the molecular basis and mechanisms of gene regulation, signal transduction, ion transport, osmoregulation and mineral nutrition will be helpful in developing selection strategies for improving salinity tolerance in crop plants.

Screening and selecting for salt tolerance

Screening large numbers of genotypes for salinity tolerance in the field is difficult, due to spatial heterogeneity of soil chemical and physical properties, and to seasonal fluctuations in rainfall. A field study in Syria using ICARDA's advanced *durum* breeding lines indicated that significant genetic variation for salt tolerance might exist, but the confounding presence of drought stress made it difficult to identify genotypes with salt tolerance (Srivastava and Jana, 1984). They concluded that 'the lack of reliable large-scale field screening techniques still seems to be the biggest problem in genetic improvement of salt and drought tolerance of crop plants'. Screening techniques that can be carried out under controlled environments have therefore often been used, especially for evaluating germination and survival of young seedlings at high salinity (200-300mM NaCl) (Munns and James, 2003).

The physiological effects of salinity on plant growth are not fully known, measuring salinity tolerance is difficult, and little is known about genes involved in salinity tolerance. Because of this complex nature of salinity tolerance, trait-based selection criteria are recommended for screening techniques (Noble and Rogers, 1992; Yeo and Flowers, 1986; Yeo *et al.*, 1990). Salinity tolerance is usually assessed as the percent biomass production in saline versus control conditions over a period of time (Munns, 2002). Salt tolerance can also be assessed in terms of survival, which is quite appropriate for most of the perennial and annual crop species. Physiological traits used for screening germplasm for salinity tolerance have included proline accumulation, Na^+ exclusion (Garcia *et al.*, 1995), K^+/Na^+ discrimination (Asch *et al.*, 2000) and Cl^- exclusion (Rogers and Noble, 1992). Relative reductions in yield and growth

have also been used as measures of plant salinity stress response. Since salinity imposes an environmental restraint on plant growth, quantitative parameters of these growth and yield reductions can be measured using the principles of biometrics and quantitative genetics. Given that the physiological approach for identifying traits that confer stress resistance so far has not been very successful (*i.e.* there is no single physiological trait that is strongly associated with salt tolerance), studying the pattern of protein synthesis under salt stress may help to identify a protein(s) associated with stress. Under saline conditions there is a change in the pattern of gene expression, and both qualitative and quantitative changes in protein synthesis.

Pearl millet and salinity

Pearl millet (*Pennisetum glaucum* [L.] R. Br.), an important cereal of traditional farming systems in tropical and subtropical Asia and sub-Saharan Africa, is the sixth most important cereal crop after wheat, rice, maize, barley and sorghum, in terms of annual global production (FAO, 1992). It is the staple food grain and a source of feed, fodder, fuel and construction material grown on 29 million ha (FAO, 2005) supporting millions of poor rural families in the drought prone semi-arid regions of Africa and the Indian sub-continent, where rainfed agriculture is practiced. Taxonomically, pearl millet belongs to the family *Poaceae*, subfamily *Panicoideae*, genus *Pennisetum*. This genus is comprised of over 140 species, with chromosome numbers in multiples of $x = 5, 7, 8$ and 9 and ploidy ranging from diploid to octaploid levels (Brunken, 1977). Sexual and apomictic, as well as annual and perennial species are included in this genus. Pearl millet is a diploid possessing $2n = 2x = 14$ chromosomes. It is a cross-pollinated annual C_4 crop species with a protogynous flowering habit, and can be intercrossed with a large group of wild relatives having $2n = 14$ chromosomes (Jauhar, 1981; Liu *et al.*, 1994b). Pearl millet was domesticated along the Southern margins of the Saharan central highlands at the onset of the present dry phase some 4000-5000 years ago (Anand Kumar, 1989). According to the earliest archeological records, pearl millet originated in Africa and was introduced to India about 2000 B.C. It is the fourth most important cereal crop in India, after rice, wheat and sorghum, and is most widely grown in

the states of Rajasthan, Maharashtra, Gujarat and Haryana. In India, the average annual area sown to pearl millet amounts to 9.5 M ha with an average annual grain production of 8.3 M tons and average grain yield of 880 kg/ha (FAO, 2005). The cultivated crop and its wild progenitor are diploid with seven pairs of large chromosomes and a haploid DNA content of 2.5 pg (Bennett, 1976). The genome size of pearl millet is around 2300 million base pairs of DNA, which is about 5 times larger than that of rice (430 M bp) and almost equal to that of maize (2400 M bp). The genome size of pearl millet is also larger than that of sorghum (750 M bp) (Arumuganathan and Earle, 1991).

Pearl millet tolerates drought, low soil fertility, and low soil pH, yet responds well to favorable water and soil conditions. Indeed, in some of the hottest and driest regions of India and Africa, where other crops do not grow well, pearl millet is the only cereal that can be grown reliably and so plays a critical role in food security. Generally, pearl millet is considered more efficient in utilization of soil moisture and has a higher level of heat tolerance than sorghum or maize. These facts make pearl millet an important food staple in rainfed regions of sub-Saharan Africa and the Indian sub-continent, especially in the semi-arid areas, where other crops tend to fail because of inadequate rainfall and poor soil conditions (FAO and ICRISAT, 1996). In recent times, there is a renewed interest globally in growing pearl millet because of its drought tolerance and high quality grain.

Although pearl millet is one of the most drought tolerant of all domesticated cereals (Bidinger and Hash, 2004), its grain yields are limited by the poor inherent fertility and water-holding capacity of the marginal soils on which the crop is largely grown, and traditional management practices (including little use of fertilizers and below optimum levels of tillage) in these stress-prone agricultural production areas. Further limitations are imposed both by salinity and drought stresses. The crop suffers from water deficits at critical growth phases, especially during crop establishment and reproductive growth. Soil salinity hampers pearl millet productivity to a great extent by delaying seed germination and reducing germination percentage, and severely affecting subsequent growth throughout the plant life cycle (Ashraf and Idrees, 1992).

Thus, research for increasing the salt tolerance of pearl millet will not only increase the productivity, but also allow more effective use of poor quality irrigation water in salt-affected areas. Additionally, the ability to grow high return crops such as pearl millet on salt-affected land will boost farm incomes and support changed farm management practices to address salinization in the semi-arid tropics.

Until recently, little breeding for salinity tolerance was undertaken in pearl millet because of lack of understanding of the tolerance mechanisms and plant responses to this stress, and of the genetic variability available for improving these. Conventional breeding for quantitative traits such as salinity tolerance is often an extremely slow and laborious process and because of genotype x environment interactions, the application of results from such breeding efforts tends to be location specific. However, recent advances in the application of DNA markers and plant genome mapping technology offers a new opportunity for understanding the genetics of salt-resistance genes and their contribution to plant performance under salinity stress. Molecular genetic maps have been developed for major crop plants, which make it possible for scientists to tag desirable traits using known DNA landmarks. Molecular markers allow breeders to track genetic loci controlling salt stress tolerance without having to measure the phenotype, thus, reducing the need for extensive field-testing over time and space. Moreover, gene pyramiding or introgression can be done more precisely using molecular tags. Together, molecular markers offer a new strategy known as marker-assisted selection (MAS), which may provide a powerful tool to improve salinity tolerance. Therefore, by identifying genomic regions that contribute most to control of salinity stress tolerance and providing molecular selection criteria can help efficiently move these genomic regions from poorly adapted trait donors into the best available improved varieties via a conventional crossing program. However, pearl millet has less sequenced genomes and a shortage of markers and there is every need to develop molecular markers for salinity tolerance in this important crop.

Taking cognizance of this background information, the present work has been taken up with the following objectives:

* **To screen a set of genetically diverse pearl millet inbred lines for their salinity tolerance levels.**

* **To assess possible mechanisms of salinity tolerance in pearl millet.**

* **To measure the antioxidant enzyme activity responses to salinity stress in salt-tolerant and -sensitive lines of pearl millet.**

* **To develop TRAP (Target Region Amplified Polymorphism) markers for salinity tolerance in pearl millet.**

* **To check the validity of molecular markers identified in the course of the study.**

* **To identify QTLs for salinity tolerance in mapping populations of pearl millet.**

The aim of this study is to assess opportunities for using existing pearl millet mapping populations (Hash and Witcombe, 1994; Hash *et al.*, 2001) and other pearl millet genetic stocks available at ICRISAT to map TRAP (Target Region Amplified Polymorphism) markers for genomic regions contributing to salinity stress tolerance. The TRAP markers thus developed and mapped may find use in genome characterization, tagging desirable genes, and high-throughput mapping of pearl millet populations.

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

2.1 Salinity – a major problem hampering crop productivity

Salinity of arable land is an increasing problem of many irrigated, arid and semi-arid areas of the world where rainfall is insufficient to leach salts from the root zone, and salinity is a significant factor in reducing crop productivity (Francois and Maas, 1994). Soil salinity is a major constraint to food production because it limits crop yields and restricts use of land previously cultivated. The United Nations Environment Program (UNEP) estimates that approximately 20% of agricultural land and 50% of cropland in the world is salt-affected (Flowers and Yeo, 1995). Much of the world's land is not cultivated, but a significant proportion of cultivated land is salt-affected. The term *salt-affected* refers to soils that are saline, and according to the *FAO Land and Plant Nutrition Management Service*, these cover over 400 million ha, which is over 6% of the world land area (Table 2.1) (FAO, 2005) Areas affected by soil salinity are not well defined, since detailed maps are available for only a few. Consequently, global estimates vary widely (Flowers *et al.*, 1986). Of the current 230 million ha of irrigated land world-wide, 45 million ha are salt-affected (19.5 %) and of the 1,500 million ha under dryland agriculture, 32 million are salt-affected to varying degrees (2.1 %). Together, these salinity-affected lands (which includes about one-third of the land area world-wide) (Figure 2.1) constitute a major constraint to food production (Singh and Chatrath, 2001).

2.2 Types and Causes of Salinity

2.2.1 Natural or primary salinity

Primary salinity results from the accumulation of salts over long periods of time, through natural processes, in the soil or groundwater. It is caused by two natural processes. The first is the weathering of parent materials containing soluble salts. Weathering processes break down rocks and release soluble salts of various types, mainly chlorides of sodium, calcium and magnesium, and to a

Table 2.1 Regional distribution of salt-affected agricultural soils, in million hectares

Regions	Total land area	Saline soils	
	Mha	Mha	%
Africa	1,899	39	2.0
Asia, the Pacific and Australia	3,107	195	6.3
Europe	2,011	7	0.3
Latin America	2,039	61	3.0
Near East	1,802	92	5.1
North America	1,924	5	0.2
Total	12,781	397	3.1%

[Source: FAO Land and Plant Nutrition Management Service, FAO 2005]

Figure 2.1. Global distribution of salt-affected soils (shown in red)

[Source : Singh and Chatrath, 2001]

lesser extent, sulphates and carbonates. Sodium chloride is the most soluble salt. Another type of salinity occurs in coastal areas subject to tides and the main cause is intrusion of saline water into rivers (Cyrus *et al.*, 1997) or acquifers (Howard and Mullings, 1996). Coastal rice crops in Asia, for instance, are frequently affected by exposure to seawater brought in by cyclones (Sultana *et al.*, 2001).

2.2.2 Secondary salinization or human-induced salinity

Secondary salinization results from human activities, mainly as a consequence of improper methods of irrigation. The most common causes are (i) land clearing and replacement of perennial vegetation with annual crops, and (ii) irrigation schemes using salt-rich irrigation water or having insufficient drainage. Szabolcs (1992) estimated that 50% of all irrigated schemes are salt-affected. Too few attempts have been made recently to assess the degree of human-induced secondary salinization and according to Flowers and Yeo (1995) this makes it difficult to evaluate the importance of salinity to future agricultural productivity. Prior to human activities, in arid or semi-arid climates, the water used by natural vegetation was in balance with the rainfall, with the deep roots of native vegetation ensuring that the water tables were well below the surface. Clearing and irrigation changed this balance, so that rainfall on one hand, and irrigation water on the other, provided more water than the crops could use. The excess water raises the water table and mobilizes salts previously stored in the subsoil, bringing them up to the root zone. Plants use the water and leave the salt behind until the soil water becomes too salty for further water uptake by roots. The water table continues to rise, and when it comes close to the surface, water evaporates leaving salts behind on the surface, thus forming a 'salt scald'. The mobilized salt can also move laterally to water courses and increase their salinity.

2.3 The Effect of Salinity on Plants

Salts in the soil water may inhibit plant growth for two reasons. First, the presence of salt in the soil solution reduces the ability of the plant to take up water, and this leads to reduction in the growth rate (Romero-Aranda *et al.*,

2001; Ghoulam *et al.*, 2002). This is referred to as the osmotic or water-deficit effect of salinity. Second, if excessive amounts of salt enter the plant in the transpiration stream there will be injury to cells in the transpiring leaves and this may cause further reductions in growth. This is called the salt-specific or ion-excess effect of salinity (Greenway and Munns, 1980). The initial and primary effects of salinity, especially at low to moderate concentrations, are due to its osmotic effects (Munns and Termaat, 1986; Jacoby, 1994). Osmotic effects of salts on plants are a result of lowering of the soil water potential due to increasing solute concentration in the root zone. At very low soil water potentials, this condition interferes with the plant's ability to extract water from the soil and maintain turgor. At high salinity, some specific symptoms of plant damage may be recognized, such as necrosis and leaf tip burn due to Na^+ or Cl^- ions (Wahome *et al.*, 2001). Sodium and chloride, usually the most prevalent ions in saline soils or water, account for most of the deleterious effects that can be related to specific ion toxicities (Levitt, 1980). The degree to which growth is reduced by salinity differs greatly with species and to lesser extent with varieties (Bolarin *et al.*, 1991; Ghoulam *et al.*, 2002).

2.4 Salt Tolerance

Flowers *et al.* (1977), Levitt (1980) and Shannon *et al.* (1994) classified plants into halophytes and glycophytes, depending on their sensitivity to salinity. Halophytes are plants that can grow in the presence of high concentrations of salts, even higher than that of seawater (~500 mM) and have a competitive advantage over non-halophytes in highly saline environments. Glycophytes on the other hand, are plants that are sensitive to relatively low salt concentrations. Almost all major crop species as well as most wild plant species are glycophytes. Although individual responses to high salinity levels may differ, several lines of evidence suggest that all plants use the same general salt tolerance regulatory mechanisms, and that the differences between halophytic and glycophytic species are of a quantitative rather than qualitative nature (Greenway and Munns, 1980; Zhu, 2001a). Plant sensitivity to salt levels in the soil is also highly dependent on environmental factors (Shannon *et al.*, 1994),

plant species, and cultivars within a species (Greenway and Munns, 1980, Ashraf, 2002), as well as the stage of plant development (Vicente *et al* , 2004)

2.4.1 Influence of growth stage on salinity tolerance

The response of plants to salinity varies with growth stage at which salinization is initiated However, information about the salt tolerance of crops at different stages of growth is limited Plant sensitivity to soil salinity continually changes during the growing stages (Vicente *et al* , 2004) The available data generally agree that the early seedling stage of plant growth is the most sensitive for most crops (Maas and Poss, 1989, Vicente *et al* , 2004) It is during this stage of growth of cereal crops that leaf and spikelet primordial are initiated and tiller buds are formed (Maas and Grieve, 1990) Consequently, high soil salinity during this crop growth stage can severely affect final economic yield Significant and non significant associations between salinity tolerance at the germination stage and adult plant growth and development were identified (Lovato *et al* , 1994, Bayuelo-Jimenez *et al* , 2002) Most crops are tolerant during germination, but the young developing seedlings are susceptible to injury during emergence from the soil and during early juvenile development Once established, plants generally become increasingly tolerant during later stages of vegetative growth, although reproductive growth may again be sensitive to salinity stress (Khatun and Flowers, 1995, Khatun *et al* , 1995)

One of the primary effects of salt stress is that it delays germination and seedling emergence Seed germination is usually the most critical stage in crop establishment, determining the success or failure of crop production (Almansouri *et al* , 2001) Earlier vegetative growth stages are more sensitive to salinity than subsequent ones (Lal, 1985) The fact is that subsequent growth and final yield of crop plants decrease when the moisture supply is limited Germination and seedling growth are reduced in saline soils with varying responses for species and cultivars (Bliss *et al* , 1986, Hampson and Simpson, 1990) Salinity affects the germination of seeds by creating an external osmotic potential that prevents water uptake or via the toxic effects of Na^+ and Cl ions on the germinating seed (Redmann, 1974, Murrillo-Amador *et al* , 2002, Khajeh- Hosseini *et al* , 2003) Although different stages of plant growth were

seen to respond differentially to salinity stress (Vicente *et al.*, 2004), in certain reports the performance of seedlings under saline conditions has been considered highly predictive of the response of adult plants to salinity (Azhar and McNeilly, 1987). Ashraf *et al.* (1986) evaluated seedlings of barley, wheat and seven forage grass species, and demonstrated considerable tolerance of salinity at the adult growth stage of these plants. Similarly, in studies conducted by Bayuelo-Jimenez *et al.* (2002), five accessions of *Phasoelus filiformis* previously identified as the most tolerant at germination and early seedling growth, were also tolerant during the vegetative growth stage when exposed to 180 mM NaCl. Hence, salinity tolerance observed at germination, early seedling and vegetative growth stages is of great importance.

2.5 Mechanisms of salt stress tolerance

Plants have adapted a variety of mechanisms to alleviate the negative impacts of salinity (Gorham, 1995a,b). Such mechanisms range from cellular level to whole plant reactions and are often an integrated response at multiple levels. Salt tolerance can be achieved by the ability of growing cells of a plant to avoid high ion concentrations (avoidance) or the ability of cells to cope with high ion concentrations (tolerance) (Greenway and Munns, 1980; Levitt, 1980). Examples of salt avoidance mechanisms include delayed germination or maturity until favorable conditions prevail; the exclusion of salt at the root zone or preferential root growth into non-saline areas; compartmentalization of salt into and secretion from specialized organelles such as salt glands and salt hairs; or storage in older leaves. According to Munns (2002), the salt tolerance mechanisms in plants fall into two main categories: those involved in minimizing salt entry into the plant and those involved in minimizing salt concentration in the cytoplasm. Thus, research into crop salt tolerance has been conducted at different levels of organization from molecular to crop level.

2.5.1 Selective accumulation or exclusion of ions

Under saline conditions, both glycophytes and halophytes either restrict the excess salts in the vacuole or compartmentalize the ions in different tissues to

minimize their disruption of metabolic functions (Zhu, 2003). Salt can be prevented from entering the plant through its root system, or within the plant salt can be restricted from reaching sensitive organs (Larcher, 1980). Internal exclusion mechanisms can involve processes such as sequestering salt ions in specialized tissues after removing them from the transport stream (Hagemeyer, 1987). Some plants rid their systems of salt by excreting it back into the environment through their roots, shoots and leaves (Larcher, 1980). Some halophytic species have evolved specialized structures in the epidermis for exclusion such as 'bladder hairs' or 'salt glands' (Hagemeyer, 1987). Bladder hairs are structures on leaf surfaces that consist of several 'stalk cells' and a 'bladder cell'. The stalk cells transport ions into the vacuole of the bladder cell, which eventually dies and falls off the plant. Salt glands are specialized structures that transport ions directly out of the plant through both roots and leaves.

In general, exclusion mechanisms are effective at low to moderate levels of salinity, whereas ion accumulation is the primary mechanism used by halophytes at high levels, presumably in conjunction with the capacity to compartmentalize ions in the vacuole (Jeschke, 1984). Glycophytes limit sodium uptake, or partition sodium in older tissues, such as leaves, that serve as storage compartments, which are eventually abscised (Cheeseman, 1988). Apse *et al.,* (1999) reported that removal of sodium from the cytoplasm or compartmentalization of it in vacuoles is done by a salt-inducible enzyme Na^+/H^+ antiporter. Some varieties that accumulate more salt ions in their leaves under salinity stress have been categorized as 'includers' (Yeo, 1983), and reports are found in sorghum (Colmer *et al.,* 1996), rice (Lutts *et al.,* 1996a) and sugarbeet (Ghoulam *et al.,* 2002). This accumulation of salt ions could play an important role in osmotic adjustment in stressed plants if the ions were efficiently compartmentalized. The ability to regulate salt concentration in the cytoplasm through compartmentalization of ions is an important aspect of salt tolerance.

2.5.2 Synthesis of compatible solutes

Salinity stress often results in the accumulation of low-molecular weight compounds in the cytosol and organelles to counterbalance the osmotic gradient created by accumulation of salts in the vacuole, or excessive salt levels in the external medium (Rhodes and Hanson, 1993;Hasegawa *et al.,* 2000a). These low molecular weight compounds are termed 'compatible solutes' as they do not interfere with normal physiological and biochemical processes in plant cells (Bohnert and Jensen. 1996). Typically, compatible solutes are hydrophilic, giving rise to the view that they could substitute for water at the surface of proteins, protein complexes, or membranes. Hence, they may act as osmoprotectants (Shinozaki and Yamaguchi-Schinozaki, 1997). Some compatible osmolytes are essential elemental ions, such as K^+, but the majority of them are organic solutes. Compatible solute accumulation as a response to osmotic stress is a ubiquitous process in organisms as diverse as bacteria and plants and animals. However, the solutes that accumulate vary with the organism and even between plant species. Compatible solutes have the capacity to preserve the activity of enzymes that are in saline solutions. These compounds have minimal effects on pH or charge balance of the cytosol or luminal compartments of organelles. The synthesis of compatible osmolytes is often achieved by diversion of basic intermediary metabolites into unique biochemical reactions. Furthermore, many of the osmoprotectants enhance stress tolerance of plants when expressed as transgene products (Bohnert and Jensen, 1996; Zhu, 2001a). Adaptive biochemical functions of osmoprotectants include scavenging of reactive oxygen species that are by-products of hyperosmotic and ionic stresses, and serving as chemical chaperones that directly stabilize membranes and/or proteins (Bohnert and Jensen, 1996; Lee *et al.,* 1997; Hare *et al.,* 1998; Bohnert and Shen, 1999). Major categories of organic osmotic solutes consist of simple sugars (mainly fructose and glucose), sugar alcohols (glycerol and methylated inositols such as mannitol, sorbitol, pinitol), and complex sugars (trehalose, raffinose and fructans) (Bohnert and Jensen, 1996; Parida *et al.,* 2002). Others include quaternary amino acids and their derivatives (proline, glycine betaine, proline betaine) (Kavi Kishor *et al.,* 2005), and tertiary amines (1,4,5,6-tetrahydro-2-methyl-4-carboxyl pyrimidine),

and sulfonium compounds (choline sulfate, dimethyl sulfonium propironate) (Nuccio *et al.,* 1999; Mansour, 2000; Sangam *et al.,* 2005). Over-expression of compatible solutes in transgenic plants can result in improved stress tolerance (Sangam *et al.,* 2005).

Proline accumulation is one of the most frequently reported modifications induced by water and salt stresses in plants and is often considered to be involved in stress tolerance mechanisms, although its precise role still remains a controversial subject. Cytoplasmic accumulation of this amino acid is thought to be involved in osmotic adjustment of stressed tissues (Delauney and Verma 1993, Kavi Kishor *et al.* 1995). It was reported that under saline conditions proline levels increase significantly in leaves of rice (Lutts *et al.*, 1996b) and in sugar beet (Ghoulam *et al.,* 2002). The proposed functions of proline under salt stress conditions include osmotic adjustment, protection of enzymes and membranes, and acting as a reservoir of energy and nitrogen for utilization during exposure to salinity (Bandurska, 1993; Perez-Alfocea *et al.,* 1993; Kavi Kishor *et al.,* 2005). Studying the effects of stress on enzyme activities involved in proline metabolism could provide valuable information on the physiological significance of its accumulation. In plants, proline is synthesized from glutamate and ornithine. Proline biosynthesis from glutamate is a function of two genes encoding Δ^1-pyrroline-5-carboxylate reductase and Δ^1-pyrroline-5-carboxylate synthetase (Delauney and Verma 1993). In plants, proline is synthesized from glutamate via Δ^1-pyrroline-5-carboxylate (P5C) by two successive reductions, which are catalyzed by P5C synthetase (P5CS; EC 2.7.2.11/1.2.1.41) and P5C reductase (P5CR; EC 1.5.1.2). Genes encoding these two enzymes have been identified in several plant species and all have been reported to be up-regulated in response to salinization (Hare and Cress, 1997; Hare *et al.,* 1998, 1999). Hence, these two enzymes form an important part of salt stress response in plants.

2.5.3 Ion homoeostasis or control of ion uptake

Homoeostasis can be defined as the tendency of a cell or an organism to maintain an internal steady state in response to any environmental perturbation or stimulus tending to disturb normality. Typically, ions

constantly move in and out of cells in a controlled fashion with net flux adjusted to accommodate cellular requirements, thus creating ionic homoeostasis. High salt concentration in the external solution of plant cells causes an ionic imbalance (Niu *et al.*, 1995; Zhu *et al.*, 1997). The intracellular concentration of Na^+ and Cl^- ions increases and disturbs the homoeostasis of these ions along with other ions like K^+ and Ca^{2+} (Serrano *et al.*, 1999; Hasegawa *et al.*, 2000a,b). As a result, plant survival and growth will depend on adaptations that re-establish ionic homoeostasis, thereby reducing the duration of cellular exposure to ionic imbalance. Plant cells respond to salt stress by increasing Na^+ efflux at the plasma membrane and Na^+ accumulation in the vacuole. Compartmentation of Na^+ and Cl^- in the vacuole is considered to be important in ameliorating the effects of salt on plants, and increasing Na^+ transport into the vacuole has been shown to increase salt tolerance (Apse *et al.*, 1999). The uptake of K^+ by plant cell is also affected by high external Na^+ concentration, due to chemical similarities between these two ions. Na^+ competes with K^+ for uptake through common transport systems and does this effectively since the external Na^+ concentration, $[Na^+]_{ext}$ in saline environments is usually considerably greater than the external K^+ concentration. Thus, plants respond to elevated external Na^+ concentrations to maintain low cytosolic Na^+ concentrations and a high cytosolic K^+/Na^+ ratio.

2.5.4 Induction of antioxidative enzymes by salinity

When plants are subjected to stresses, such as salt stress, high concentrations of reactive oxygen species (ROS) are formed (Gossett *et al.*, 1994a; Hernandez *et al.*, 1995). Plants possess numerous defense mechanisms, both enzymatic and non-enzymatic, and these mechanisms contribute to protect cells from oxidative injury (Scandalios 1997; Shalata and Tal, 1998; Dionisio-Sese and Tobita, 1998; Gomez *et al.*, 1999). The main non-enzymatic antioxidants are reduced glutathione (GSH), cysteine, hydroquinones, mannitol, vitamins C and E, flavonoids, some alkaloids and β-carotene. The enzymatic antioxidant defenses include superoxide dismutase (SOD), catalase (CAT) and peroxidase. In addition, the whole array of enzymes in ascorbate-glutathione cycle [ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione

reductase (GR)] are needed for the regeneration of the active forms of the antioxidants.

Superoxide dismutase (SOD) is a major scavenger of O^{2-} and its enzymatic activity results in the formation of H_2O_2 (Beauchamp and Fridovich 1971). CAT, APX and a variety of general peroxidases then catalyze the breakdown of H_2O_2. In the ascorbate-glutathione cycle, the enzymatic action of APX produces mono-dehydro-ascorbate (MDA) that can be reduced spontaneously or enzymatically to dehydro-ascorbate (DHA) by NADPH-dependent mono-dehydro-ascorbate reductase (MDHAR). DHA is reduced back to ascorbate non-enzymatically by reduced glutathione (GSH) or enzymatically in a reaction mediated by DHAR. The resulting oxidized glutathione (GSSG) is then converted back to the reduced from by NADPH-dependent GR. CAT and SOD are the most efficient antioxidative enzymes (Scandalios, 1993). Their combined action converts the potentially dangerous O^{2-} and H_2O_2 to water and molecular oxygen, thus averting cellular damage. Within a cell, SOD constitutes the first line of defense against ROS. While all compartments of the cell are possible sites for O^{2-} formation, chloroplasts, mitochondria, and peroxisomes are thought to be the most important generators of ROS (Fridovich, 1986). Therefore, SODs are present in all these subcellular locations. Based on the metal co-factor used by the enzyme, SODs are classified into three groups: 1) iron SOD (Fe SOD), 2) manganese SOD (Mn SOD), and 3) copper-zinc SOD (Cu-Zn SOD). Fe SODs are located in the chloroplasts, Mn SODs are located in the mitochondria and the peroxisomes, and Cu-Zn SODs are found in the chloroplasts, the cytosol, and possibly in the extracellular space (Grene, 2002). All three groups of SOD enzymes are encoded in the nucleus, and SOD genes have been shown to be sensitive to environmental stresses, presumably as a consequence of increased ROS formation.(Parida *et al.,* 2004a,b).

Upon the imposition of oxidative stress, the existing pool of reduced glutathione (GSH) is converted to oxidized glutathione (GSSG) and glutathione biosynthesis is stimulated (May and Leaver, 1993; Madamanchi *et al.,* 1994). Glutathione reductase (GR) activities increase as the glutathione pool increases through a multi-level control mechanism, which includes coordinated activation of genes

encoding glutathione biosynthetic enzymes and GR (Xiang and Oliver, 1998). Glutathione is a potent cellular reductant with a broad redox potential. It acts as a scavenger of peroxides and serves as a storage and transport form of reduced sulphur (May *et al.,* 1998). Due to the redox active thiol group GSH may be involved in the regulation of the cell cycle and can act as a defence compound against oxidative stress. GSH has been shown to participate in the regeneration of the reduced form of ascorbate through non-enzymatic reduction of DHA at an alkaline pH (Noctor *et al.*, 1998). Glutathione has also been reported to regulate the induction of antioxidant defenses, as exemplified by the induction of Cu-Zn SOD (Herouart *et al.,* 1993). Glutathione *S*-transferases (GSTs) are another set of well-characterized enzymes involved in stress tolerance (Marrs, 1996). These enzymes catalyze the conjugation of glutathione to a wide variety of electrophiles including cytotoxic substrates (Mannervik and Danielson, 1988; Pickett and Lu, 1989).

2.5.5 Influence of plant hormones and Ca^{2+} on salinity

Several phytohormones can reduce the salt-induced inhibition of plants and alleviate the deleterious effects of salinity. Of these, gibberellins have been the main focus of published studies (Chakrabarti and Mukherji, 2002; Angrish *et al.*, 2001; Basalah and Mohammad, 1999; Kozlowski, 1997; Munjal and Goswami, 1995). For instance, treatment with gibberellic acid (GA_3) has been reported to be helpful in enhancing wheat growth under saline conditions (Parasher and Varma, 1988; Ashraf *et al.*, 2002).

Salinity also interacts with plant nutrients and decreases plant Ca^{2+} levels (Al-Harbi, 1995). Franco *et al.* (1999) suggested that Ca^{2+} could have a protective effect in root tips, which is of fundamental importance for the maintenance of root elongation in NaCl-stressed seedlings. Calcium protects plants from the adverse effects of NaCl salinity and improves the growth of plants under saline conditions (Cramer *et al.,* 1990). There are a number of reports that show that increasing the Ca^{2+} concentration in growth media, increases germination of seeds of different crops in NaCl solutions, including wheat (Chaudhuri and Wiebe, 1968), maize (Alberico and Cramer, 1993) and rice (Lin and Kao, 1995).

6 Pearl millet: current status and future potential

Of all the world's cereals, pearl millet (*Pennisetum glaucum* (L.) R. Br.) is the sixth most important. Descended from a wild West African grass, it was domesticated more than 4,000 years ago, along the Southern margins of the Saharan central highlands at the onset of the present dry phase (Anand Kumar, 1989). Long ago, it spread from its homeland to East Africa and then to India. Both places adopted it eagerly and it became a staple food crop. Pearl millet is grown principally for grain in the tropical and sub-tropical areas of Africa and the Indian subcontinent. It is planted on ~15 million ha in Africa and ~11 million ha in South Asia (mostly in India), yielding annually ~10 million tons of grain (Anand Kumar, 1989). Pearl millet is member of a genus with over 140 species, with chromosome numbers in multiples of X = 5, 7, 8 and 9, and ploidy levels ranging from diploid to octaploid (Brunken, 1977). Both sexual and apomictic species, as well as annual and perennial species, are included in this genus. The cultivated crop and its wild progenitor are diploid with seven pairs of large chromosomes and a haploid DNA content of 2.5pg (Bennett, 1976).

India is a major pearl millet producing country with 43.4 per cent of the world area and 42 per cent of world pearl millet grain production. Five states, Rajasthan, Maharashtra, Gujarat, Uttar Pradesh and Haryana account for nearly 90 percent of the total cultivated area under pearl millet (9.5 million ha) in India (FAO, 2005). Generally, pearl millet is considered the best-adapted rainy season cereal crop for arid and semi-arid conditions because of its more efficient utilization of soil moisture and higher level of heat tolerance than sorghum and maize (Harinarayana *et al.*, 1999).

2.6.1 Uses of pearl millet

In its traditional growing areas, pearl millet grain is the basic staple for households in the poorest countries and among the poorest people. The stalks are also used for hay, pasture, silage, building material, and fuel (Baker, 2003). The grain can be consumed like rice in sweet or savoury dishes, or can be ground into a powder and used as flour for making bread, porridge, etc (Facciola, 1990). The sweet tasting nutritious grains are eaten raw by children

and the grain is often fermented to make various foods and alcoholic beverages (Facciola, 1990). In the Sahel and elsewhere in Northern Africa, pearl millet is an important ingredient of couscous. Pearl millet stalks are a valued building material, fuel and livestock feed. The plant is medicinally useful as an appetiser and tonic, and also in the treatment of heart diseases (Chopra *et al.*, 1986). In non-traditional growing areas in the USA, Brazil, Australia, and Europe, pearl millet is grown as both a forage and grain crop. The forage is used by cattle and other livestock. The grain is used to feed cattle and poultry birds and domesticated pets. Pearl millet grain is showing promise as a feedstock for ethanol production also.

2.6.2 Nutritive value of pearl millet grain

2.6.2.1 Food Value

As a cereal for human food, pearl millet grain is considered to be highly palatable and is among the most nutritional of grain crops. The protein content is not only high but it is also of good quality. The protein content of pearl millet varies from 8 to 23 per cent, lysine from 0.9 to 3.8 per cent, oil 2.8 to 8.0 per cent, and carbohydrates 59.7 to 74.5 per cent (Jambunathan, 1980) and provides about 360 Kcal 100g^{-1} energy. The amino acid profile of pearl millet grain is better than that of sorghum and maize and is comparable to that of wheat, barley and rice (Ejeta *et al.*, 1987) with a less disparate leucine/isoleucine ratio (Hoseney *et al.*, 1987; Rooney and McDonough, 1987). As reported by ICAR (2006), pearl millet has protein (11.3-19.6%), starch (35.7%), total sugars (2.0-2.7%), fat (3.0-4.6%) and also has good amounts of phosphorous and iron. The special nutritional quality of its grain particularly for micronutrients such as iron and zinc content facilitates its use in health food formulations and fits well in value addition (Sagar and Kumar, 2005, pers. commun.).

2.6.2.2 Feed Value

Pearl millet grain has a good potential to be used as a high quality feed grain like corn and sorghum in rations of chickens, cattle and swine (Hoseney *et al.*, 1987; Smith *et al.*, 1989; Serna-Saldivar *et al.*, 1990; Hancock *et al.*, 1990;

Hanna *et al*, 1991) The energy density of pearl millet grain is relatively high, arising from its higher oil content relative to maize, wheat or sorghum (Hill and Hanna, 1990) Feeding tests on cattle, swine, and particularly chickens have shown pearl millet grain is at least equivalent to maize and often superior to sorghum in feed rations, generally because of its high energy and grain protein levels (Rooney and McDonough, 1987, Sullivan *et al*, 1990, Bramel Cox *et al* 1992) Pearl millet does not contain any condensed polyphenols, such as the tannins in sorghum, which can interfere with digestibility (Andrews *et al*, 1993)

2.6.3 Importance of pearl millet for research

Pearl millet is well adapted to production systems characterized by low rainfall, low soil fertility, and high temperature, and thus can be grown in areas where other cereal crops, such as rice, wheat or maize, would not survive Its combination of rapid growth rate when conditions are favorable, high temperature tolerance, and ability to extract mineral nutrition and water from even the poorest soils make it impossible to beat in the world s harshest agricultural production environments Information on the genetics of different traits of any crop is important for the systematic breeding and long term improvement With its low chromosome number, availability of an impressive range of variation for several morphological characters, ease of selfing and deliberate crossing, production of both selfed and crossed seed in good quantities, relatively short life cycle and more importantly adaptation to adverse climatic conditions, pearl millet is amenable to genetic studies (Vinchon, 1949, Burton and Powell, 1968) Despite its importance, however, pearl millet can be considered a lost crop because its untapped potential is still vast Currently, this grain is an orphan among the cereals In fact, few people outside of India and parts of Africa have ever heard of it As a result, it lags behind sorghum and far behind the other major grains in its genetic development Further, grain yields of pearl millet are limited by the poor inherent fertility and water-holding capacity of the soils on which it is grown and traditional management practices, including little use of fertilizers and below-optimum levels of tillage Limitations are also imposed by salt and drought stresses In environments where pearl

millet is cultivated recurrent droughts, soil salinity, insect pests, diseases, and the root parasite *Striga*, are common Another major production constraint is the low grain yield potential of traditional land race varieties

2.6.3.1 Breeding

The floral morphology, breeding behavior and the structure of grain yield make pearl millet one of the most flexible and responsive crop species to breed A very wide range of genetic variability is available in the primary germplasm pool for improvement of this species where genetic manipulation is facilitated by its tillering protogynous habit and high seed number per panicle With the correct selection of parent lines in regard to phenotype and relative maturity, hybrids can also be made in pearl millet by utilizing the natural period of protogyny This method allows quicker hybrid development, greatly increases the range of possible parent combinations, and avoids diseases that are associated, particularly in Africa, with the use of cytoplasmic male-sterile seed parents

2.6.3.2 Molecular mapping in pearl millet

Molecular mapping has proved a vital and promising tool in the on-going battle to improve pearl millet In 1994, the first RFLP-based genetic map of the pearl millet genome was produced (Liu *et al*, 1994b) and within a year scientists were able to map genes that conferred resistance to downy mildew, which is the most important disease of this crop Since that time, more than 600 molecular markers were developed and mapped, and using marker-assisted selection, additional disease resistance and drought tolerance genes have been incorporated into elite hybrid parental lines to strengthen the crop s natural resistance to diseases and tolerance to abiotic stresses The first product of such marker-assisted selection "HHB 67 Improved" was released for cultivation in 2005 (Gazette of India, 2005, Hash *et al*, 2004)

2.6.4 Pearl millet and salinity tolerance

Pearl millet is one of the two most widely cultivated drought-tolerant C_4 cereals that are grown under rainfed and dryland conditions in drought-prone regions of the tropics and subtropics It is especially important as a staple food grain,

Jaswant S. Kanwar Library
ICRISAT
BR 63786

and source of feed and fodder for livestock, in the marginal agricultural production environments of Africa and South Asia that are home to hundreds of millions of the world's poorest crop-livestock producers (ICRISAT and FAO, 1996). Salinity stress is an important abiotic constraint to production of this crop, and is a major contributor to the instability of its grain and fodder yields (as shown in Figure 2.2). The growth of pearl millet in saline medium is severely affected at different stages of the plant life cycle (Ashraf and McNeilly, 1987; Ashraf and Idrees, 1992). Ashraf and Idrees (1992) noted that salinity stress caused reduction in the germination percentage and delayed germination of seeds of pearl millet. Until recently, little breeding for drought and salt tolerances was undertaken because of lack of understanding of the tolerance mechanisms and plant responses to these stresses. Since, pearl millet is a cereal crop with less sequenced genome and a dearth of markers, there is every need to develop molecular markers for salt and drought tolerances in this important crop.

Tools and knowledge already developed within other cereal crops can also greatly benefit pearl millet. Establishing the genetic similarities between grass species allows transfer of information from one crop to another. Genes found to control drought tolerance in rice, for example, may predict those genes that play an important role in pearl millet. Comparative mapping of rice, foxtail millet and pearl millet demonstrated the genomic relationship between these crops and, importantly, pearl millet is seen as a 'bridge' between various cereals (Devos *et al*, 2000).

2.7 Molecular marker analysis

The development of molecular markers for physiological traits has made significant headway in recent years with the advancement of new technologies. Consequently, the use of molecular markers in breeding programs is increasing rapidly as they have been shown to greatly improve the efficiency of breeding programs for traits for which conventional phenotypic selection is difficult, expensive and/or time-consuming. Molecular markers are rapidly being adopted by crop improvement researchers globally as an effective and

Figure 2.2. Effect of saline soil on establishment and growth of a pearl millet hybrid in Rajasthan, India (2004)

[Note: Only a handful of plants were able to survive in this salt-affected field corner.]

[Photograph courtesy Dr C T Hash, ICRISAT, Patancheru, India.]

appropriate tool for basic and applied studies addressing biological components in agricultural production systems (Jones *et al.*, 1997; Mohan *et al.*, 1997; Prioul *et al.*, 1997). The ability to score genotypes at the molecular level provides a huge increase in the available markers for any analysis. The technology is capable of handling large numbers of samples.

PCR-based molecular markers have the potential to reduce the time, effort and expense often associated with phenotypic screening. The first molecular markers used were isozymes, which are protein variants detected by differences in migration on starch gels in an electric field (Stuber and Goodman, 1983). The limitation with protein markers lies with insufficient protein variation for high-resolution mapping (Burrow and Blake, 1998). However, as methods for evaluating variation directly at the DNA level became widely available during the mid-1980s, DNA-based markers replaced isozymes in mapping studies. A significant breakthrough in genetic analysis came when the first genetic map using restriction fragment length polymorphisms (RFLPs) (Botstein *et al.*, 1980) was constructed. Since then molecular biology has ushered in a new era with techniques that directly assay DNA and overcome many of the problems that have previously limited the applied use of biochemical markers.

2.7.1 DNA markers

DNA markers are alleles of loci at which there is sequence variation or polymorphism in DNA. Such variation is often, but not always, neutral in terms of phenotype (Jones *et al.*, 1997). These markers have the advantage in that they are not influenced by the environment, are expressed in all tissues and can be scored at all stages of plant growth. Because of this, DNA markers have proved valuable in crop breeding; especially in studies on genetic diversity within plant species and mapping of regions of the genome that contribute to variation in traits of economic importance. DNA markers may be broadly divided into three classes based on the method of their detection: (1) hybridization-based; (2) polymerase chain reaction (PCR)-based and (3) DNA sequence-based (Winter and Kahl, 1995; Jones *et al.*, 1997; Gupta *et al.*, 1999; Joshi *et al.*, 1999). DNA markers reveal genetic differences that can be visualized by using a combination of gel electrophoresis and staining with

chemicals (ethidium bromide or silver) or detection with radioactive or colorimetric probes. Recently, Mohan *et al.* (1997), Kumar (1999) and Gupta and Varshney (2000) extensively reviewed the details for these markers systems. The references for these different marker systems are given in Table 2.2. A whole array of DNA-based markers is now available including RFLPs (restriction fragment length polymorphisms), RAPDs (randomly amplified polymorphic DNAs) and AFLPs (amplified fragment length polymorphisms) and more recently simple sequence repeats (SSRs) or microsatellites (Staub *et al.*, 1996; Gupta and Varshney, 2000). These polymorphic markers provide the framework maps around which QTLs (Quantitative Trait Loci) can be located. The advantages and disadvantages of the most commonly used markers are presented in Table 2.3 (Collard *et al.*, 2005).

Of the rapid PCR-based marker techniques, RAPD is a simple method to fingerprint genomic DNA, but poor consistency and low multiplexing output limit its use. AFLPs are now widely used for a variety of applications due to their high multiplexing ratio, reproducibility and ease of use (Vos *et al.*, 1995). The main disadvantage of this method is however, its complexity, being necessary to perform multiple steps including DNA digestion, ligation and amplification, which make it difficult to optimize the conditions for each step. Simple sequence repeats (SSRs) [also known as variable number tandem repeats (VNTRs), simple tandem repeats (STRs), sequence tagged microsatellite sites (STMS) or microsatellites] are generally among the most reliable and highly reproducible of molecular markers, forming the foundation for many framework linkage maps. Although SSRs have the advantage of providing mostly co-dominant markers, the technique can require considerable investment to generate the necessary primer sequences, since this requires sequence information from more conserved flanking regions, which is expensive and time-consuming to generate. The large start-up costs for this technique should be justifiable for crops where large-scale mapping and MAS are a practical necessity (Hash and Bramel-Cox, 2000). In short, the major limitations of the available methods are low reproducibility of RAPD markers; high cost and complexity of generating AFLP markers; and the expense and time-consuming protocols, and limited number of loci detected per PCR reaction for SSR

Table 2.2 Molecular marker techniques

(Source: Mohan *et al.*, 1997; Gupta and Varshney, 2000)

Acronym	Techniques	References
AFLP	Amplified Fragment Length Polymorphism	Vos *et al.* (1995)
ALP	Amplicon Length Polymorphism	Ghareyazie *et al.* (1995)
AP-PCR	Arbitrarily Primed PCR	Welsh and McClelland (1990)
AS-PCR	Allele-Specific PCR	Sarkar *et al.* (1990)
CAPS	Cleaved Amplified Polymorphic Sequence	Lyamichev *et al.* (1993)
DAF	DNA Amplification Fingerprinting	Caetano-Anolles *et al.* (1991)
IMP	Inter-MITE (Miniature Inverted-repeat Transposable Elements) Polymorphism	Chang *et al.* (2001)
ISA	Inter-SSR Amplification	Ziekiewicz *et al.* (1994)
MP-PCR	Microsatellite-Primed PCR	Meyer *et al.* (1993)
RAMS	Randomly Amplified MicroSatellite	Ender *et al.* (1996)
RAPD	Random Amplified Polymorphic DNA	Williams *et al.* (1990)
REMAP	Retrotransposon-Microsatellite Amplified Polymorphism	Kalendar *et al.* (1999)
RFLP	Restriction Fragment Length Polymorphism	Botstein *et al.* (1980)
SAP	Specific Amplicon Polymorphism	Williams *et al.* (1991)
SCAR	Sequence Characterized Amplified Region	Williams *et al.* (1991)
SRAP	Sequence Related Amplification Polymorphism	Li and Quiros (2001)
SNP	Single Nucleotide Polymorphism	Nikiforov *et al.* (1994)
SSCP	Single-Strand Conformation Polymorphism	Orita *et al.* (1989)
SSLP	Simple Sequence Length Polymorphism	Saghai *et al.* (1994); Jarman and Wells (1989)
SSR	Simple Sequence Repeats	Hearne *et al.* (1992)
STMS	Sequence Tagged Microsatellite Sites	Beckmann and Soller (1990)
STS	Sequence Tagged Sites	Fukuoka *et al.* (1994)

Table 2.3 Advantages and disadvantages of the most commonly used DNA markers for QTL analysis

(Source: Collard *et al.*, 2005)

Molecular marker	Codominant (C) or Dominant (D)	Advantages	Disadvantages	References
RFLP	C	• Robust • Reliable • Transferable across populations	• Time-consuming, laborious and expensive • Large amounts of DNA required • Limited polymorphism (especially in related lines)	Beckmann & Soller (1986), Kochert (1994), Tanksley *et al.* (1989)
RAPD	D	• Quick and simple • Inexpensive • Multiple loci from a single primer possible • Small amounts of DNA required	• Problems with reproducibility (usually low) • Generally not transferable	Penner (1996), Welsh & McClelland (1990), Williams *et al.* (1990)
SSR	C	• Technically simple • Robust and reliable • Transferable between populations • Allele-specific	• Large amounts of time and labour required for production of primers • Usually require PAGE • Limited in number for high density map construction	McCouch *et al.* (1997), Powell *et al.* (1996), Taramino & Tingey (1996)
AFLP	D	• Multiple loci • High levels of polymorphism generated	• Large amounts of DNA required • Complicated methodology	Vos *et al.* (1995)

markers. However, high-throughput marker technologies are needed for more rapid and reliable mapping of plant genomes to identify genomic regions harbouring genes governing desirable traits like salt and drought tolerance, and then exploit these in applied marker-assisted selection programmes. Recently, Li and Quiros (2001) reported a simple molecular marker technique called Sequence Related Amplification Polymorphism (SRAP), which aims for the amplification of open reading frames (ORFs). The SRAP technique uses pairs of primer with AT- or GC- rich cores to amplify intragenic fragments for polymorphism detection. The common feature of this technique with AFLP and RAPD is that multiple fragments can be generated in a single PCR reaction, making them more efficient than SSR markers. However, these earlier techniques do not use prior sequence information, and the markers generated are randomly distributed across the genome. They can be used jointly with bulked segregant analysis (BSA) (Michelmore *et al.*, 1991) to screen for markers linked to desirable agronomic traits.

Access to increasing numbers of EST sequences obtained from diverse cDNA libraries coupled with freely available bioinformatic tools allows us to explore new opportunities in crop molecular research for providing markers to targeted regions of the genome. The recently developed rapid and efficient PCR-based technique known as TRAP (target region amplification polymorphism) possibly opens up new avenues to overcome the limitations of the previously described marker techniques. While random DNA markers are derived from polymorphic sites genome wide, gene-targeted markers are derived from polymorphisms within genes and thus reflect functional polymorphism (Andersen and Lubberstedt, 2003). Hence, gene-targeted markers like TRAPs (Hu and Vick, 2003), may be more meaningful than random DNA markers as they could directly contribute to variation of the concerned trait under study.

2.7.1.1 Target Region Amplification Polymorphism (TRAP)

The Target Region Amplification Polymorphism (TRAP) technique employs an 'arbitrary' 18-mer primer in combination with a 'fixed' 18-mer primer designed based on known expressed sequence tag (EST) sequences to amplify genomic fragments around targeted candidate genes (Hu and Vick, 2003). The arbitrary

primer of about 18 nucleotides is designed with either an AT- or GC-rich motif to anneal with an intron or exon, respectively (Hu and Vick, 2003; Li and Quiros, 2001). The fixed primer, also about 18 nucleotides long, is designed from EST sequences of genes of interest obtained from the freely accessible online databases. PCR amplification is run for the first 5 cycles with an annealing temperature of 35°C, followed by 35 cycles with an annealing temperature of 50°C. Each PCR reaction can generate as many as 50 scorable fragments with sizes ranging from 50-900 bp when separated on a 6.5% polyacrylamide sequencing gel. Reproducibility has been a concern for easily generated markers, such as RAPDs (Jones *et al.,* 1997; Virk *et al.,* 2000). Because TRAPs use longer primers than RAPDs, they have better reproducibility, an advantage over RAPDs (Hu and Vick, 2003). Further, an advantage claimed for both RAPDs and AFLPs over allele-specific PCR markers is that prior sequence information is not needed to generate markers. However, the TRAP technique now takes the advantage of the availability of sequence information, using the known partial sequence of a candidate gene as the fixed primer and an arbitrary primer to amplify regions associated with the putative candidate gene and similar genes throughout the genome. Also, TRAP detects a large number of loci in a single PCR reaction without extensive pre-PCR processing of samples, which is an advantage over the time-consuming and expensive SSRs that detect only a few loci in a single reaction (Liu *et al.,* 2005) and over AFLPs that require considerable pre-PCR processing. These advantages of TRAP markers suggest that they should offer many advantages for plant genomics research involved in marker-trait association, high density map construction, detecting quantitative trait loci and genotyping for genetic diversity studies.

Since it was first reported by Hu and Vick (2003), the TRAP marker technique has been applied to germplasm characterization, genetic variability assessment among cultivars, and genome mapping of various crop species like wheat (Xu *et al.,* 2003; Liu *et al.,* 2005), bean (Miklas *et al.,* 2004, 2006), lettuce (Hu *et al.,* 2005), sunflower (Hu *et al.,* 2004,; Rojas-Barros *et al.,* 2005) and sugarcane (Arro, 2005; Alwala *et al.,* 2003, 2006). Xu *et al.* (2003) used TRAPs to characterize genetic stocks of tetraploid wheat and found that a large number of

chromosome-specific markers could be generated with this technique. TRAPs were found useful for tagging and mapping disease resistance traits in common bean (Miklas *et al.,* 2006). Use of the TRAP protocol to develop markers associated with sunflower downy mildew resistance showed that it was possible to detect the presence of downy mildew fungus and to genotype host plants in the same PCR reaction (Hu *et al.,* 2004). Liu *et al.* (2005) used SSRs and TRAPs to generate over 700 markers for the construction of a genetic linkage map in a hard red spring wheat intervarietal recombinant inbred population, indicating that TRAPs are highly efficient for genetic mapping in wheat. The applicability of the TRAP marker technique to lettuce genotyping was also demonstrated very recently (Hu *et al.,* 2005).

2.7.2 Linkage mapping

A linkage map may be thought of as a 'road map' of the chromosomes derived from two different parents (Paterson, 1996). Linkage maps indicate the position and relative genetic distances between markers along chromosomes, which are analogous to signs or landmarks along highways. The most important use of linkage maps is to identify chromosomal locations containing genes and QTLs associated with traits of interest; such maps may then be referred to as 'QTL' (or 'genetic') maps. Construction of a linkage map is the most fundamental step required for a detailed genetic study and application of the marker-assisted breeding approach in any crop (Tanksley *et al.,* 1989). Comprehensive mapping of QTLs requires informative markers for all regions of the nuclear genome (Paterson *et al.,* 1988; Lander and Botstein, 1989). Likewise, a high-density map facilitates marker-assisted selection, especially between progeny of closely related parents (Chittenden *et al.,* 1994), as it provides information on many potentially polymorphic markers in all genomic regions. Genetic maps show the order of loci on a chromosome and the relative distance between them. Such maps are essential for localization of genes affecting both simple and complex traits. Construction of linkage maps is based on the discovery that Mendelian factors or genes controlling inheritance are organized in a linear order on chromosomes. Sturtevert developed the first chromosome map using segregation data from studies on *Drosophila* (Crow and Dove, 1988). Later

chromosome maps in several organisms were developed. The markers on these maps were either genes controlling simply-inherited morphological variants or morphological features of the chromosomes themselves. Until recently, construction of chromosome maps proceeded slowly because of limited polymorphism in morphological markers, and the large amounts of time and labour consumed in construction of marker stocks and in genetic mapping by indirect observation of recombinant chromosomal segments (Kochert, 1994). The principle of construction of molecular maps is same as in classical genetic mapping. However, the new consideration in molecular mapping is that a potentially unlimited number of DNA markers can be analyzed in a single mapping population (Young, 2001). Parents selected for mapping experiments should show sufficient polymorphism for both phenotypic characters and molecular markers. This cannot be over-emphasized, for in the absence of DNA polymorphism, segregation analysis and linkage mapping would be impossible (Young, 2001). TRAP markers tend to exhibit high levels of polymorphism, providing the possibility of constructing maps in crosses between even closely related plants.

Since the resolution of a linkage map and the ability to correctly determine marker order is largely dependent on population size, the decision on population size to be used for mapping is critical. Whenever it is possible, a larger population is better (Young, 2001). Based on Monte Carlo simulations, Beavis (1994) concluded that populations smaller than 200 individuals would rarely be successful to find most QTLs and in many cases populations larger than 500 are required. More over, if the goal is high resolution mapping in specific genomic regions or mapping QTLs with minor effect, a much larger population is required (Young, 2001).

For mapping projects the most widely used genetic mapping software is MAPMAKER (Lander *et al.*, 1987). MAPMAKER is based on the concept of the LOD score, "the log of odds ratio" (Morton, 1955). The computer program JOINMAP is especially suited to relate one's map to those derived from other mapping populations (Stam, 1993).

2.7.2.1 Linkage maps in pearl millet

Detailed genetic linkage maps in plants are very useful tools for studying genome structure and evolution, identifying introgression between genomes, and localizing genes of interest. RFLP markers have simple genetic segregation patterns and are potentially unlimited in number. Detailed RFLP linkage maps were constructed for several crops such as maize, tomato, lettuce, potato and rice. In pearl millet, the development of molecular marker system and molecular marker-based genetic maps was initiated early, in 1990, within a Department for International Development (DFID)-funded program involving several UK laboratories and breeders at the ICRISAT, Patancheru. The first RFLP-based genetic linkage map of pearl millet was reported by Liu *et al.* (1994b). Initial work to develop a molecular marker-based genetic linkage map of pearl millet was reported by Liu *et al.* (1992, 1994a,b). This began with a focus on the use of RFLP markers detected using homologous pearl millet probes, with ^{32}P-mediated autoradiography to visualize banding differences. A few heterologous probes from rice, wheat and barley were also included in this base map. The map contained 181 Restriction Fragment Length Polymorphism (RFLP) marker loci that covered a genetic distance of 303 cM (Kosambi units) containing all the seven linkage groups. This map probably corresponds to the centromeric regions of the seven pearl millet chromosome pairs. It was used to map QTLs for resistance to pearl millet downy mildew by Jones *et al.* (1994, 1995). This initial map was transferred to several additional crosses (Busso *et al.,* 1995; Liu *et al.,* 1996) for studies of sex-specific recombination rates in cultivated-cultivated and cultivated-wild crosses, and a pearl millet world reference mapping population was developed based on the cross of 81B and ICMP 451 (Hash and Witcombe, 1994). This map has since been used for saturation genotyping using SSR markers (Breese *et al.,* 2002; Qi *et al.,* 2004), and RFLPs based on additional homologous probes from pearl millet and heterologous probes from other grasses. The latter group of markers has improvied our understanding of the complex relationships between pearl millet genome and those of other cultivated graminaceous species (Devos and Gale, 1997; Devos *et al.,* 2000). This work has extended the total pearl millet genetic linkage map length to approximately 600 cM (Hash and Bramel-Cox, 2000).

Recently, genetic linkage maps of four different pearl millet crosses were integrated to develop a consensus map of about 353 RFLP (220 homologous and 133 heterologous RFLP markers) and 65 SSR markers (Qi *et al.,* 2004). An interesting feature of the genetic maps of pearl millet is the extreme localization of recombination towards the chromosome ends. The concentration of mapped markers in centromeric regions, reflecting an unequal distribution of recombination, was first observed in the early molecular maps of wheat (Chao *et al.,* 1989) and has since been seen in several species (Devos *et al.*, 1992; Qi *et al.,* 1996; Tanksley *et al.,* 1992), but this appears to be extreme in pearl millet. Physical mapping of one such region on linkage group 1 revealed a physical mapping to genetic distance ratio of <12 kb/cM (Padi and Devos, unpublished). This unequal distribution of recombination appears to be largely cross-independent, and will have consequences for the transfer of traits from agronomically inferior donors to elite pearl millet germplasm. The integration of markers previously mapped in other grass species has provided the anchor points to align the pearl millet linkage groups to other cereal genetic maps, including the cereal model, rice. Although the pearl millet genome appears to be relatively highly rearranged relative to rice, regions of colinearity between the two species can be clearly identified (Devos *et al.,* 2000). These now form a framework for exploitation of the rice genomic sequence as a source of new markers and candidate genes underlying traits in pearl millet. A pearl millet mapping population derived from a cross between ICMB 841 and 863B was studied for DNA polymorphism to construct a genetic linkage map, and to map genomic regions associated with grain and stover yield, and aspects of drought tolerance (Hash *et al.,* 2003; Yadav *et al.,* 2004). The genetic map length and the distribution of markers for this population were comparable to the consensus map of pearl millet (Devos *et al.,* 2000) and to other maps published for this species (Jones *et al.,* 1995; Yadav *et al.,* 2002). The microsatellite loci were added subsequently to the RFLP markers being mapped due to their utility in subsequent marker-assisted selection. These also displayed a clustering of loci in the centromeric regions with very few loci mapping to the distal regions of the chromosomes. Compared to the better-studied cereals such as rice, wheat, maize and barley, there has been relatively little research on the development

and application of molecular genetic maps of pearl millet (Hash *et al.*, 2003). In spite of the huge global pearl millet germplasm collections, only a few of them were analyzed for the genetic diversity due to non-availability of less tedious marker system for this crop. A subset comprising of 504 landrace accessions from the global pearl millet germplasm collections was recently assessed phenotypically for genetic diversity by Bhattacharjee *et al.* (2002) and a subset of 10 accessions were then characterized for RFLP allelic diversity by examining 51 loci in 25 plants per accession. Genetic relationships among the 10 accessions were similar, whether based on morphological characters or RFLP allelic constitution. Genotype identification and assessment of genetic relationships in pearl millet were carried out using microsatellites and RAPDs by Chowdari *et al.* (1998). Pearl millet belongs to the class of less sequenced genomes and it still has dearth of PCR-compatible molecular markers for the construction of a high density map. Some attempts were made to develop SSR markers for this crop (Qi *et al.*, 2001, 2004; Allouis *et al.*, 2001; Budak *et al.*, 2003; Senthilvel *et al.*, 2004). Recently, Bertin *et al.* (2005) developed a new type of markers namely; single stranded conformational polymorphism - single nucleotide polymorphism (SSCP-SNP) markers, for this crop. However, all these new markers are still not sufficient to construct a high-density map for pearl millet. Genetic linkage maps have been developed for various pearl millet crosses and used to detect and map QTLs contributing to various traits. Information on the position of QTLs relative to marker loci provides a basis for marker-assisted selection (MAS) for quantitative traits.

2.7.3 Marker-trait associations

Quantitative characters have been a major area of genetic study for over a century because they are a common feature of natural variation in populations of all eukaryotes (Kearsey and Farquhar, 1998). First attempts at studying them stemmed from the work of Galton (1889) on man before the rediscovery of Mendellian inheritance of quantitative characters through the pioneering work of Fischer (1918), Wright (1934), Mather (1949) and Falconer (1989) to the new era. Despite these studies, the number of genes and their interactive effects controlling the expression of quantitative traits are poorly understood. In plants

the first attempts to use markers to perform genome-wide analysis of quantitative variation used allozymes (Tanksley *et al.*, 1982; Edwards *et al.*, 1987). Later RFLPs were used as DNA markers (Beckmann and Soller, 1983; Lander and Botstein, 1989), but these were followed by PCR markers such as RAPDs, microsatellites and AFLPs that were cheaper, safer and provided more marker data per unit of DNA (Westman and Kresovich, 1997). These polymorphic markers provided the framework maps around which the polygenes/QTLs could be located (Kearsey and Farquhar, 1998). It is well understood that G × E interactions exist for many quantitative traits, suggesting that general conclusions about QTLs, particularly those with small effects detected on the basis of single environments and single populations could lead to erroneous decisions. The use of QTL identification by breeders also will be influenced by the consistency of QTL regions across the germplasm (Bubeck *et al.*, 1993). One challenge of plant breeding is to take advantage of favourable direct effects of QTLs, while maximizing favourable environmental interactions and minimizing unfavorable ones (Bubeck *et al.*, 1993).

2.8 QTL mapping

QTL analysis is predicated on looking for associations between the quantitative trait and the marker alleles segregating in the population. It has two essential stages: the mapping of the markers and association of the trait with the markers. Both of these require accurate data and statistical software (Kearsey and Farquhar, 1998). The theory of QTL mapping was first described in 1923 by Sax, where he noted that seed size (a complex trait) in common bean was associated with seed coat color (a simple, monogenic trait). This concept was further elaborated by Thoday (1961), who suggested that if the segregation of simply inherited monogenes could be used to detect linked QTLs, then it should eventually be possible to map and characterize all the QTLs involved in complex traits. Modern QTL mapping is essentially the fulfillment of this idea, with the key innovation being that defined sequences of DNA act as the linked monogenic markers. With the development of comprehensive DNA marker maps (Phillips *et al.*, 1994), it is now possible to search for QTLs throughout the genomes of the most economically important crop species. This has had the

profound effect of moving the focus in studies of polygenic traits to questions about the chromosomal locations, gene actions, and biological roles of specific loci involved in complex phenotypes. In simple terms, QTL analysis is based on the principle of detecting an association between phenotype and the genotype of markers. QTL mapping involves testing DNA markers throughout a genome for the likelihood they are associated with a QTL. Individuals in a suitable mapping population [F_2, backcross (BC), recombinant inbred lines (RILs)] are analyzed in terms of DNA marker genotypes and the phenotype of interest. For each DNA marker, the individuals are split into classes according to marker genotype. Markers are used to partition the mapping population into different genotypic groups based on the presence or absence of a particular marker allele and to determine whether significant differences exist between groups with respect to the trait being measured (Tanksley, 1993; Young, 1996). Mean and variance parameters are calculated and compared among the classes. A significant difference between classes (phenotypic means of the groups) suggests that there is a relationship between the DNA marker and the trait of interest, *i.e.* the DNA marker is probably linked to a QTL (Munns *et al.*, 2002).

While the concept of QTL mapping seems clear and simple, there are still many limitations in practice. Many DNA marker maps are not sufficiently dense to achieve the potential of QTL mapping, since sparse marker maps severely limit the power of QTL mapping (Lander and Botstein, 1989). Even under optimal experimental conditions, multiple QTLs on a single linkage group are difficult or impossible to resolve. Populations must be relatively large in order to uncover minor loci, and the biological relevance of loci uncovered depends on the cut-off chosen for statistical significance (Lander and Botstein, 1989). Since the traits of interest are, by nature, genetically complex, environmental factors and genetic background potentially have an enormous impact on results. Of course, this is one of the most powerful applications of QTL mapping (*i.e.* analyzing gene × gene and gene × environment interactions), but it also means that many large, time-consuming experiments need to be carried out to analyze a system thoroughly. An alternative approach using multiple regressions was developed by Haley and Knott (1992). It often produces very similar results to LOD mapping both in terms of accuracy and precision, but has the advantages of

speed of calculations and simplicity of programming. Tests of significance and confidence intervals can be obtained. In studies of complex traits such as salinity tolerance, factors all the way from use of a suitable screening technique to difficulties in quantitative assessment of salt tolerance make QTL mapping more challenging. Fortunately, powerful computer software programs are now available to analyze QTL mapping results (Lander and Botstein, 1989; Lincoln *et al.*, 1993; Basten *et al.*, 1994, 2001; Utz and Melchinger, 1996, 2000, 2003), to handle hundreds of markers simultaneously (Young, 2001) and better DNA marker systems are being developed to simplify the technique and increase marker density (Zabeau, 1993).

2.8.1 Methods to detect QTLs

Three widely used methods for detecting QTLs are single-marker analysis, simple interval mapping and composite interval mapping (Liu, 1998; Tanksley, 1993). Single-marker analysis (also referred to as single-point analysis) is the simplest method for detecting QTLs associated with single markers. The statistical methods used for single-marker analysis include *t*-tests, analysis of variance (ANOVA) and linear regression. Linear regression is commonly used because the coefficient of determination (R^2) for the marker is equivalent to the portion of phenotypic variation arising from the QTL linked to the marker (provided that there is very tight linkage between the marker and the QTL). This method does not require a complete linkage map and can be performed with basic statistical software programs. However, the major disadvantage with this method is that the further a QTL is from a marker, the less likely it will be detected because of increased likelihood of the occurrence of recombination between the marker and the QTL. This causes the magnitude of the effect of a QTL to be under-estimated (Tanksley, 1993). The simple interval mapping (SIM) method makes use of linkage maps and analyses intervals between adjacent pairs of linked markers along chromosomes simultaneously, instead of analyzing single markers (Lander and Botstein, 1989). The use of linked markers for analysis compensates for recombination between the markers and the QTL, and is considered statistically more powerful compared to single-point analysis (Lander and Botstein, 1989; Liu, 1998). Many researchers used

MapMaker/QTL (Lincoln *et al.,* 1993) and QGene (Nelson, 1997) to conduct SIM. Recently, composite interval mapping (CIM) has become popular for mapping QTLs. This method combines interval mapping with linear regression and includes additional genetic markers in the statistical model in addition to an adjacent pair of linked markers for interval mapping (Jansen, 1993; Jansen and Stam, 1994; Zeng, 1993, 1994). The advantage of CIM is that it is more precise and effective at mapping QTLs compared to single-point analysis and interval mapping, especially when linked QTLs are involved. For use of linkage information in marker-assisted breeding, a program like Map Manager (Manly and Cudmore, 1998) helps to keep track of marker data in the population of interest. Hypergene (Young and Tanksley, 1989) or Graphical Genotyper (GGT) can help to display graphical genotypes. The program qGENE seeks to bring all of these important DNA marker tools together into single package (Nelson, 1997). Many workers use QTL Cartographer (Basten *et al.,* 1994, 2001), MapManager QTX (Manly *et al.,* 2001) and PLABQTL (Utz and Melchinger, 1996, 2000, 2003) to perform CIM.

2.8.2 QTL mapping in pearl millet

QTL mapping and DNA markers have provided insights into facets of quantitative inheritance patterns in pearl millet. QTLs for host-plant resistance to downy mildew were identified from parental line IP 18293 for six Indian and two African downy mildew pathogen populations of pearl millet (Azhaguvel, 2001). A downy mildew resistance QTL on pearl millet LG4 was found linked to the d2 dwarfing gene generated from a cross between PT 732B containing the major d2 dwarfing gene and P1449-2, the donor of the resistance QTL (Padi *et al.,* 2001). Mapping of QTLs involved in the domestication syndrome traits for this crop was realized using two F_2 populations derived from crosses of domesticated (*Pennisetum glaucum* ssp. *glaucum*) × wild (*Pennisetum glaucum* ssp. *monodii*) pearl millet (Poncet *et al.,* 2002). In another study to identify specific genomic regions associated with the enhanced tolerance of pearl millet to drought stress during the flowering and grain-filling stages, several QTLs associated with traits determining grain and stover yield were mapped (Yadav *et al.,* 2002). QTLs were mapped for foliar disease resistance (Morgan *et al.,* 1998),

downy mildew resistance (Jones *et al.,* 1994, 1995, 2002; Kolesnikova-Allen, 2001; Breese *et al.,* 2002; Azhaguvel *et al.,* 2003; Hash and Witcombe, 2002; Gulia, 2004), drought tolerance (Yadav *et al.,* 2002, 2004), and genotype × environment interactions of flowering time, and grain and stover yield under favorable conditions (Yadav *et al.,* 2003), for stover yield and quality parameters (Hash *et al.,* 2003) and for characters involved in domestication of this crop (Poncet *et al.,* 2000, 2002). Marker-assisted backcross programs to transfer the target QTLs associated with downy mildew resistance, terminal drought tolerance, and stover yield and quality parameters into parental lines of popular pearl millet hybrids are well underway in ICRISAT. However, progress is slow, mainly because of non-availability of simple-to-use PCR compatible markers to cover the entire pearl millet genome. Nonetheless, the first non-transgenic product of marker-assisted selection (MAS) to be released for cultivation in India was pearl millet hybrid "HHB 67 Improved", which has as its male parent a product of marker-assisted backcrossing with improved downy mildew resistance in the background of H77/833-2, the male parent of the original HHB 67 (Gazette of India, 2005).

2.8.3 QTL mapping for salinity tolerance

Quantitative traits for stress tolerance, which are likely to be expressed under stress and which show large environmental effects, need a screening procedure designed to cope with the expected degree of variation. Like other quantitative traits, salinity tolerance in cereals is polygenic. Phenotypic selection for such traits is difficult. Selection based on markers could theoretically ease the manipulation of such traits without affecting other agronomic traits. At the genetic level, salinity tolerance is considered to be a quantitative trait (Foolad and Jones, 1993) and has generally proven recalcitrant to attempts to improve it by conventional plant breeding. Understanding the physiology of salinity tolerance is critical to the identification of QTLs and thereby flanking molecular markersthat could be used for MAS. Study of salt tolerance of sorghum based on assessment of tolerance to NaCl as relative root length in salt treated as compared with control plants, showed that there were both additive and dominance effects of NaCl (Azhar and McNeilly, 1989). Several authors tried to

estimate the number of loci associated with salt tolerance of barley at germination and at the seedling stage by using a composite cross population (Jana *et al.,* 1980), isogenic lines (Mano and Takeda, 1995; Mano and Takeda, 1996) or doubled haploid lines (Mano and Takeda, 1996). However, the loci controlling salt tolerance could not be determined, probably because of insufficient marker information for locating genes. Subsequently, QTLs controlling salt tolerance at germination and early seedling growth stages in barley were identified by interval mapping analysis using marker information from two doubled haploid populations (Mano and Takeda, 1997b). The map positions were different for the QTLs controlling salt tolerance at germination and at early seedling growth stages, indicating that salt tolerance during germination and subsequent seedling growth were controlled by different loci. QTLs for salt tolerance at germination in barley were reported by Dadshani *et al.,* (2004). Earlier, the K^+/Na^+ discrimination locus *Kna1* was mapped in wheat (Dubcovsky *et al.,* 1996). QTLs independently governing Na^+ and K^+ uptake and $Na^+:K^+$ selectivity associated with component physiological traits determining salt tolerance were reported in rice (Koyama *et al.,* 2001). In the absence of adequate candidate genes for salt tolerance, a QTL/MAS approach using putative AFLP markers for ion transport and selectivity identified QTLs for ion uptake in rice (Flowers *et al.,* 2000). Selection for Na^+ exclusion in wheat revealed favorable QTL alleles predominantly from the low-Na^+ uptake parent (Munns *et al.,* 2002). Thus, QTLs for salt tolerance have been described in several cereal species, including barley (Ellis *et al.,* 1997; Mano and Takeda,1997b), wheat (Semikhodskii *et al.,* 1997; Munns *et al.,* 2002; Lindsay *et al.,* 2004) and rice (Flowers *et al.,* 2000; Prasad *et al.,* 2000; Koyamo *et al.,*2001; Yao *et al.,* 2005). However, these studies did not yield robust markers that can be used across a range of germplasm, significant associations between the trait and the markers reported being confined to the populations in which they were derived.

There are numerous other reports in literature on QTLs identified in several crops controlling various traits related to salinity tolerance (Koornneef and Stam, 2001). For example, for Na^+/K^+ ratios in tissues of rice subjected to salinity stress (Gregorio, 1997); locus for sodium exclusion (*Nax 1*) in wheat

(Lindsay *et al.,* 2004); salt-tolerance in tomato (Foolad and Jones, 1993; Fooland and Chen, 1999; Fooland *et al.,* 1999, 2001; Fooland, 2004); salt tolerance at germination and the seedling stage in barley (Mano and Takeda, 1997b); effects of salinity on vegetative growth of *Arabidopsis* (Quesada *et al.,* 2002), etc. Some success has been reported in breeding methods employing marker-assisted selection. For example, Stuber (1995) reported the production of enhanced hybrids of maize some of which out-yielded check hybrids by more than 15% through the use of DNA-based markers. Cho *et al.* (1994) used molecular markers to select for the semi-dwarf characteristics in rice. In tomato, Frary *et al.* (2000) used molecular markers to identify QTL alleles that increased fruit size and successfully introgressed one QTL into large fruited cultivars. However, reports on such successful applications of QTL mapping or marker-assisted selection for salinity stress tolerance in pearl millet are lacking. Therefore, the development of high-density DNA maps that incorporate large numbers of molecular markers and use of advanced marker-assisted selection techniques to facilitate pyramiding traits of interest is essential to attain substantial improvement in salt tolerance of pearl millet.

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

3.1 Seed Materials

Twenty-eight inbred pearl millet genotypes (ICMP 85410-P7, LGD 1-B-10, Tift $23D_2B_1$-P1-P5, WSIL-P8, 81B-P6, ICMP 451-P8, ICMP 451-P6, H 77/833-2-P5(NT), H 77/833-2, PRLT 2/89-33, W 504-1-P1, P310-17-Bk, PT 732B-P2, P1449-2-P1, ICMB 841(=841B)-P3, 863B-P2, IP 18293-P152, Tift $238D_1$-P158, Tift 186, Tift 383, ICMB 89111, ICMB 90111, ICMB 92666, ICMB 95333, 843B, ICMB 98004, ICMB 99022, and ICML 22,) obtained from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India were tested for salt stress tolerance during germination and early seedling growth over salt concentrations ranging from 0mM to 200mM NaCl. The first 18 of these 28 inbred lines are parental pairs of nine ICRISAT pearl millet mapping population progeny sets; Tift 186 and Tift 383 are forage hybrid pollinators from Tifton, Georgia, USA used as control lines; and the following four inbreds are maintainer lines of male-sterile lines used as testers in line × tester trials assessing the opportunities to use the ICRISAT pearl millet mapping populations to map various secondary target traits (Hash *et al.*, 2001, 2003). The final four lines in this set are ICML 22 (Singh *et al.*, 1994), derived from an oasis landrace accession (IP 2696) from Chad that was expected to exhibit some degree of salt tolerance; 843B (Stegmeier *et al.*, 1998), and two lines near-isogenic to 843B (ICMB 98004 and ICMB 99022) derived by backcrossing ICML 22 (as the donor of oligogenic downy mildew resistance) to recurrent parent 843B (CT Hash, unpublished) [Ref. Table 3.1].

3.2 Screening of Pearl Millet Inbred Lines for Salt Stress Tolerance

The 28 inbred lines were screened for salt stress tolerance in a soil-free *in vitro* environment to reduce the complexity of genotype × environment interactions.

Table 3.1 Salient features of twenty-eight inbred lines of pearl millet tested for salt stress tolerance

Name	Pedigree/Origin	Comments	References
(ICMP 85410)-P7	{[SC 14(M)-1] × [SD2 × EB 2 (D1088)]-1}-64	d_2 dwarf, late flowering	Hash and Witcombe 1994; Liu *et al.* 1994a,b; Jones *et al.* 1995; Talukdar *et al.* 1998; Bidinger *et al.* 1999; Devos *et al.* 2000
(LGD 1)-B-10	Partial backcross d_2 dwarf, e_1 early (donor = Tift 756) derivative of a bold-seeded *Iniadi* landrace sample from Togo; bred at Tifton, GA, USA; reselected at ICRISAT-Patancheru	d_2 dwarf, e1 photoperiod-insensitive early flowering	Burton 1981; Hash and Witcombe 1994; Liu *et al.* 1994a,b; Jones *et al.* 1995; Bidinger *et al.* 1999; Devos *et al.* 2000
(Tift 23D$_2$B$_1$)-P1-P5	Partial backcross d_2 dwarf derivative of forage seed parent maintainer line Tift 23B$_1$; bred at Tifton, GA, USA	d_2 dwarf,many tillers	Burton 1969; Liu *et al.* 1994a,b
(IP 18292, WSIL)-P8	Genetic stock (*ws*, d_2, *y*, *gl*) with complex pedigree developed at ICRISAT-Patancheru	d_2 dwarf, long panicle	Appa Rao *et al.* 1990;Hash and Witcombe 1994; Liu *et al.* 1994a,b; Appa Rao *et al.* 1996; Singh *et al.* 1997
(81B)-P6	Downy mildew resistant outcross derivative of Tift 23D$_2$B$_1$ selected from a mutation breeding program at ICRISAT-Patancheru	d_2 dwarf, short panicle bristles	Anand Kumar *et al.* 1984; Hash and Witcombe 1994; Devos *et al.* 2000
(ICMP 451)-P8	Downy mildew resistant selection from restorer ICMP 451 (LCSN 72-1-2-1-1)	Tall, long panicle bristles	Hash and Witcombe 1994; Anand Kumar *et al.* 1995; Devos *et al.* 2000
(ICMP 451)-P6	Downy mildew resistant selection from restorer ICMP 451 (LCSN 72-1-2-1-1)	Tall, long panicle bristles	Hash and Witcombe 1994; Anand Kumar *et al.* 1995; Breese *et al.* 2002
H 77/833-2)-P5(NT)	Off-type segregant from H 77/833-2	Short, many tillers, photoperiod-sensitive early flowering	Kapoor *et al.* 1989; Hash and Witcombe 1994; Breese *et al.* 2002
H 77/833-2	Elite pollinator line from Haryana Agricultural University, Hisar, Haryana, India	Short, many tillers, photoperiod-sensitive early flowering, seedling heat stress tolerant	Kapoor *et al.* 1989; Hash and Witcombe 1994; Yadav *et al.* 2002, 2003
PRLT 2/89-33	Inbred line bred at ICRISAT-Patancheru from the Bold Seeded Early Composite (largely based on *Iniadi* landrace germplasm and derived breeding materials), with the pedigree BSEC 8501-13-2-2-3-2	Medium tall, early flowering; seedling heat stress sensitive; terminal drought stress tolerant	Hash and Witcombe 1994; Yadav *et al.* 2002, 2003
(W 504)-1-P1	Breeding line from Indian Agricultural Research Institute, New Delhi, India	Tall, medium-late flowering	Kolesnikova-Allen 2001
(P310-17)-B	Stable source of downy mildew resistance selected at ICRISAT-Patancheru from germplasm line IP 6329 from Mali	Tall, late flowering	Singh *et al.* 1997; Kolesnikova-Allen 2001

Table 3.1 contd.

Name	Pedigree/Origin	Comments	References
(PT 732B)-P2	"Spontaneous" dwarf mutant in elite breeding line from Tamil Nadu Agricultural University, Coimbatore, India	d_2 dwarf, photoperiod-sensitive late flowering	Appadurai *et al.* 1982; Nepolean 2003
(P1449-2)-P1	IP 21168; stable source of downy mildew resistance selected at ICRISAT-Patancheru from germplasm line IP 5853 from Senegal	Tall, photoperiod-sensitive late flowering	Singh *et al.* 1997; Nepolean 2003
(ICMB 841)-P3	Downy mildew resistant outcross of MS 5141B; developed at ICRISAT-Patancheru by pure line selection for disease resistance in a contaminated seed lot of MS 5141B	Medium tall, medium-early flowering	Singh *et al.* 1990; Yadav *et al.* 2004
(863B)-P2	Maintainer line developed at ICRISAT-Patancheru by selfing in a bold-seeded Iniadi landrace sample from Togo	Medium tall, medium-early flowering, drought tolerant	Yadav *et al.* 2004
(IP 18293)-P152	d_2 dwarf, *P* purple foliage genetic stock with complex pedigree developed at ICRISAT-Patancheru	d_2 dwarf, late flowering, poor pollen producer	Appa Rao *et al.* 1996; Singh *et al.* 1997; Azhaguvel 2001; Azhaguvel *et al.* 2003
(Tift 238D$_1$)-P158	d_1 dwarf restorer of the A_1 cytoplasmic male-sterility system bred at Tifton, GA, USA	Late flowering	Burton 1966; Appa Rao *et al.* 1986; Azhaguvel 2001; Azhaguvel *et al.* 2003
Tift 186	Forage pollinator bred at Tifton, GA, USA by selfing in a forage germplasm accession from South Africa	Tall, late flowering	Burton 1977
Tift 383	d_2 dwarf forage pollinator bred at Tifton, GA, USA from Tift 186 (Tift 239D_2B_2 × Tift 186)	d_2 dwarf, late flowering	Burton 1980
ICMB 89111	[843B × (GNS × SS-48-40-4)-1-9-8]-30-B-B-1	d_2 dwarf, many tillers; A1 cytoplasm maintainer	Rai and Rao 1998
ICMB 90111	ICMP 423 selection	Tall; EGP-261 cytoplasm maintainer	Rai *et al.* 1994
ICMB 92666	[ICMPES 34 × (843B × ICMPES 34)]-155-4-2	Tall; A_1 cytoplasm maintainer	Rai *et al.* 1998
ICMB 95333	{[(B 282 × S10B-38)-35 × Togo-29+]-53+ × {843A × [843B × (B 282 × ¾ EB)-11+]}}-60-29-1	d_2 dwarf, large panicle; A4 cytoplasm maintainer	KN Rai, pers. comm.
843B	Selection from KSU line BKM 2068	d_2 dwarf, early flowering, A1 cytoplasm maintainer	Stegmeier *et al.* 1998
ICMB 98004	Backcross derivative of 843B with one additional major gene for downy mildew resistance from ICML 22	d_2 dwarf, early flowering; A1 cytoplasm maintainer	Hash, unpublished
ICMB 99022	Backcross derivative of 843B with two or three additional major genes for downy mildew resistance from ICML 22	d_2 dwarf, early flowering; A1 cytoplasm maintainer	Hash, unpublished
ICML 22	Downy mildew resistant pure-line selection from IP 2696, an oasis landrace accession from Chad	Short, extra early flowering; A1 cytoplasm restorer	Singh *et al.* 1994

3.2.1 Materials

Test tubes made of Borosil glass (150 mm × 25 mm) plugged with non-absorbent cotton and autoclavable plastic caps were used for germination and seedling growth. Most of the inorganic salts and chemicals were obtained from Hi Media, Merck and Qualigens. Plant growth regulators were of high purity and purchased from the Sigma Inc., St. Louis, MO, USA. All other chemicals were purchased from the Fermentas Inc., Hanover, MD, USA and from Qiagen Inc., CA, USA. All the media stock solutions were prepared according to the composition of the nutrient medium using glass-distilled water. The stock solutions were stored in the refrigerator until use. Whenever hydrates of salts were used, appropriate corrections were made. The constituents of the media were added in the order shown in Appendix II. Media pH was adjusted to 6.7 with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid (as found necessary) before autoclaving. Sterilization of media, test tubes and bottles was performed by autoclaving the contents at 121 °C under 15 psi (1.05 kg cm^{-2}) pressure for 15–20 minutes. Heat labile compounds were subjected to filter sterilization using Millipore filters (0.22μm) under pressure.

All operations including inoculations and transfers were conducted in a sterile ultraviolet light treated laminar airflow transfer hood. The surface of the hood was rubbed down first with 5% soap solution, rinsed with water, dried and finally sterilized with 70% alcohol prior to each use. The interior of the chamber was saturated with an aerosol of 2% thymol and 2% glycerine in 90% ethyl alcohol. All the surgical instruments were sterilized by autoclaving before use. Spatula, inoculating loop, anatomical scissors, needles and scalpels were further sterilized by flaming with absolute alcohol inside the laminar flow cabinet. To maintain maximum sterility inside the laminar flow hood, it was swabbed with 70% ethanol and the UV lights were switched on for 10–15 minutes before every operation. All the test tube cultures were incubated under continuous white fluorescent light (30 $\mu Em^{-2}s^{-1}$) at an ambient temperature of 24 ± 2 °C.

3.2.2 Surface sterilization and germination of seeds to early seedling stage

Seeds of pearl millet inbred lines were soaked in 0.1% Bavistin solution for 30 seconds, washed with sterile distilled water and surface-sterilized with 70% ethanol for 1-2 min. Surface sterilized seeds were washed three times with sterile distilled water and germinated on filter-paper boats in balanced nutrient solutions (Hoagland and Arnon, 1938) of pH 6.7 at 20°C, containing four different concentrations of NaCl (0 mM, 75 mM, 100 mM, and 150 mM) in triplicates for each experiment. About 15 seeds were put for germination in each test tube. The experiments were repeated 4 times for each line and the data means of each experiment were taken for the studies and the statistical analysis. Seedlings from germinating seeds were allowed to grow for 10 days, at 25°C under continuous fluorescent light (30 $\mu Em^{-2}s^{-1}$). At harvest, shoots and roots of healthy seedlings were immediately separated and washed quickly with distilled water to remove any possible salt surface contamination.

3.2.3 Morphological parameters

Observations on germination efficiency (%), length (cm) of shoots and roots, root/shoot ratio, and fresh and dry weight (mg) of shoots and roots were recorded for each entry for all four screening environments (three levels of salinity and the non-stressed control treatment).

3.2.3.1 Germination efficiency (%)

Germination efficiency (%) was recorded for each entry after 10 days of seedling growth as follows:

Germination efficiency (%) = 100 × (Number of seeds germinated / Total number of seeds inoculated)

Seeds were considered germinated when the emergent radicle reached 2 mm in length.

3.2.3.2 Length of shoot and root (cm)

The shoot and root of each seedling were detached. Shoot length (cm) was measured from the base of the plant to the tip of the top-most completely opened leaf on ten randomly selected plants from each treatment using a

standard ruler/scale in centimeters. Similarly, the root length (cm) was measured from the base of the plant to the tip of the root. The mean shoot and root length for each treatment was calculated as follows:

Mean shoot/root length (cm) for each treatment = (Sum of all the ten shoots/root lengths recorded from each test tube) / 10.

The relative shoot/root lengths were also calculated as follows:

Relative shoot/ Root length = [(Shoot/Root length in saline solution) / (Shoot/Root length in control solution)] × 100

The relative lengths of shoots and roots were subjected to analysis of variance (Sigma Plot, 2001).

3.2.3.3 Root/shoot ratio

The ratio of root length (cm) to shoot length (cm) for each treatment was calculated.

3.2.3.4 Fresh and dry weight (mg) of shoots and roots

The fresh and dry weights (mg) were recorded on a Mettler balance for each of ten randomly selected shoots and roots per treatment. On the 10th day, fresh weights of radicles and hypocotyls were measured. Subsequently the radicles and hypocotyls were dried at 80°C for 24 h, and weighed. Cotyledons were not included in fresh and dry weight comparisons, since they reflect imbibition rather than growth.

The relative shoot/root lengths were also calculated as follows:

Relative shoot/root dry Wt. = [(Shoot/Root dry wt. in saline solution) / (Shoot/Root dry wt. in control solution)] × 100

The relative dry weight of shoots and roots were subjected to analysis of variance (Sigma Plot, 2001).

3.3 Biochemical Parameters

3.3.1 Estimation of proline

Proline was determined by modification of the method outlined by Bates *et al.* (1973). Approximately 0.1 g of dry weight of tissue was homogenized in 1ml of 3% aqueous sulfosalicylic acid in a chilled mortar and pestle. Neutral glass

powder was used for homogeneous grinding. Two ml of 3% sulphosalycylic acid was added followed by centrifugation at 2000 rpm, 4°C for 10 min. One ml of the supernatant was reacted with 1 ml of glacial acetic acid and 1 ml of acid-ninhydrin (2.5 g ninhydrin was dissolved in 50 ml of solvent prepared by mixing glacial acetic acid and 6M phosphoric acid) for 30 min at 100°C in a boiling water bath. The reaction mixture was terminated in an ice bath after 30 min. Four ml of toluene was added to the reaction mixture and then vigorously mixed using a cyclomixer for 15–20 seconds. The two phases were then allowed to separate and brought back to room temperature. The reactant chromophore containing toluene (upper phase) was aspirated and absorbance was read at 520 nm using toluene as a blank. Proline concentrations in the samples were determined from the standard curve calibrated with different concentrations of the standard proline. Proline content was expressed in terms of μg/mg dry weight of tissue.

3.3.2 Measurement of Na^+ and K^+ ions

Na^+ and K^+ were determined by flame photometry using 60 mg of dry weight of seedlings made to ash at 800°C.

Steps in Sample Preparation for flame photometry:

- For 60mg of the sample 5ml of HCl and 2 to 3 drops of perchloric acid were added. The samples were mixed and kept at 60–70°C for total evaporation.
- To the above treated sample, 2ml of HCl and 2 ml of HNO_3 were added and the samples were kept at 60–70°C for total evaporation.
- To the above treated sample 1 ml of HCl was added and the samples were kept at 60–70°C for total evaporation.
- Finally, the samples were dissolved in 2ml of double-distilled water. The samples must be transparent at this stage and it can be subjected to flame photometry for ion quantification.

For measuring ions such as Na^+, K^+, Ca^{2+} and Cl^- in short term salt treatments; 50 mg of dry weight of seedlings was used. Ions were extracted by boiling the dried seedlings in distilled water and incubated in a boiling water bath for an hour. Ion contents were estimated by using a Metrohm Ion Analyzer (Model No.

AGCH - 9101). Specific electrodes were used for estimations by following the instructions from the manual provided by the company. Standard solutions of Na^+, K^+, Ca^{2+} and Cl^- supplied by the company were used for calibration.

3.3.3 Salt stress induced antioxidant responses

Antioxidant responses under salt stress in five inbred lines of pearl millet, viz. ICMB 90111, WSIL-P8, 863B-P2, Tift $23D_2B_1$-P1-P5 and 841B-P3 (sensitive, sensitive, moderately tolerant, highly tolerant and highly tolerant respectively to salt stress) were studied by subjecting them to 0mM and 150mM NaCl stress for short durations of time (0, 24, 48, 72, 96, 120 and 144 h). A subset of three pearl millet lines, viz. ICMB 90111, 863B-P2 and 841B-P3 (sensitive, moderately tolerant and highly tolerant respectively to salt stress) were subjected to 0mM, 75mM, 100mM and 150mM NaCl for 7 days.

3.3.3.1 Preparation of enzyme extracts

Seedlings of pearl millet were homogenized in liquid nitrogen and dissolved in 100 mM sodium phosphate buffer (pH 7.4) containing 0.1mM EDTA, 1% (w/v) PVP and 0.5% (v/v) Triton-X 100. The homogenate was centrifuged at 15000 rpm for 20 min at 4°C. The supernatant was collected for measurement of specific activities of antioxidant enzymes and glutathione content, and stored at -20°C for further analysis. Soluble protein content in the enzyme extract was measured according to the method of Bradford (1976).

3.3.3.2 Estimation of glutathione

Reduced glutathione (GSH) was determined by Ellman's method (1959). The assay mixture consisted of 0.2 M phosphate buffer (pH 8.0), 5% (w/v) trichloroacetic acid (TCA), Ellman's reagent [prepared by dissolving 19.8 mg of 5-5'-dithio-bis (2-nitrobenzoic acid) in 100 ml of 0.1% sodium citrate] and appropriate tissue extract. The yellow colour developed after the addition of Ellman's reagent was read at 412 nm. The amount of GSH present in the tissue extract was calculated by using standards with reduced glutathione and the glutathione content was expressed in moles of GSH/g fresh wt.

3.3.3.3 Enzyme assays

The activity of superoxide dismutase (SOD) was measured based on the ability of the enzyme to inhibit the auto oxidation of pyrogallol. The measurement was based on the modified method of Marklund and Marklund (1974). The reaction mixture consisted of 0.252 M pyrogallol and an appropriate volume of the enzyme extract. The reaction was initiated by light illumination and the rate of oxidation was measured spectrophotometrically at 420 nm. Specific activity of SOD was expressed as units of SOD/mg protein. The unit is defined as the amount of the enzyme, which causes 50% inhibition of pyrogallol oxidation. The activity of catalase (CAT) was assayed from the rate of hydrogen peroxide (H_2O_2) decomposition as measured by the decrease of absorbance at 240 nm, following the modified procedure of Claiborne (1985). The reaction mixture consisted of distilled water, 0.059 M H_2O_2 and an appropriate volume of tissue extract in a final volume of 3 ml. Catalase activity was expressed as µl of H_2O_2 consumed/min/mg protein. Glutathione reductase (GR) activity was assayed following the method of Schaedle and Bassham (1977). The reaction mixture consisted of 0.1 M phosphate buffer (pH 7.4), 10 mM EDTA, 20 mM oxidized glutathione, 10 mM NADPH and an adequate quantity of tissue extract in a total volume of 2.0 ml. The enzyme activity was quantified at 25°C by measuring the disappearance of NADPH at 340 nm. Specific activity of the enzyme was expressed in n.moles of NADPH oxidized/min/mg protein. Glutathione S-transferase (GST) activity was determined by the method of Petit *et al.* (1996), using 1-chloro-2-dinitrobenzene (CDNB) as a substrate (Habig *et al.*, 1974). The assay mixture consisted of 0.2 M phosphate buffer (pH 7.4), 0.2 mM GSH and 0.2 mM CDNB in a total volume of 2 ml. The reaction was initiated by the addition of appropriate aliquot of the tissue extract. The increase in absorbance at 25°C was recorded at 340 nm and the enzyme activity is expressed in n.moles of CDNB conjugated/min/mg protein.

3.3.3.4 Lipid peroxidation

Lipid peroxidation was determined following the method of Utley *et al.* (1967) with minor modifications. Preliminarily, incubation mixture (20 µM $FeSO_4$, 400 µM ascorbate, 0.25 M KCl and 0.04 M Tris-Cl) was added to distilled water and

an appropriate volume of enzyme extract. The contents were then incubated at 37°C for 30 min. To the incubated assay mixture, 20% chilled trichloroacetic acid and 0.67% thiobarbituric acid were added. After a thorough mixing of all the contents, the samples were boiled at 100°C for 10 min and the solution was centrifuged at 5000 rpm for 5 min. The supernatant was separated after centrifugation and the absorbance read at 540 nm for the end product malondialdehyde (MDA) (a thiobarbituric acid reactive substance (TBARS), which is an index of lipid peroxidation). The content of MDA was expressed in n.moles/mg protein.

3.3.3.5 Isoenzyme studies

3.3.3.5.1 Isoenzyme activity of superoxide dismutase (SOD)

Electrophoresis was carried out at 4°C according to a modified procedure of Gabriel (1971) with a 10% polyacrylamide mini-slab gel in standard tris-glycine buffer (pH 8.3). Samples were loaded into each well and then electrophoresed at 100 V through the stacking gel for 15 min and 120 V through the separating gel for 60 min. After electrophoresis, a modified photochemical method of Beauchamp and Fridovich (1971) was used to locate SOD activities on gels. The gel was first soaked in 25 ml of 1.23 mM nitroblue tetrazolium (NBT) for 15 min, briefly washed, then soaked in the dark in 30 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 28 mM TEMED and 0.02 mM riboflavin for another 15 min. The gel was briefly washed again, and then illuminated for 15 min to initiate the photochemical reaction. All the procedures were carried out at room temperature, and the two soaking steps were shaken at 75 rpm.

3.3.3.5.2 Isoenzyme pattern of catalase

Electrophoresis was carried out at 4°C with a 10% polyacrylamide mini-slab gel in standard tris-glycine buffer (pH 8.3). Catalase activity following native-PAGE was determined as described by Vitoria *et al.* (2001). Protein (50 μg) was loaded in each gel lane and then electrophoresed at 100 V through the stacking gel for 15 min and 120 V through the separating gel for 60 min. Gels were incubated in 0.003% H_2O_2 for 10 min and then developed in a 1% (w/v) $FeCl_3$ and 1%

(w/v) $K_3Fe(CN)_6$ solution for 10 min. All the procedures were carried out at room temperature, and the two soaking steps were shaken at 75 rpm.

3.3.3.5.3 Isoenzyme activity of ascorbate peroxidase

Native-PAGE was performed using a stacking gel containing 4.3% acrylamide and a separating gel containing 7.5% acrylamide with a running buffer composed of 4 mM Tris-HCl, pH 8.3, and 38 mM glycine. In each lane, 300 µg of total proteins was loaded in each lane. After non-denaturing electrophoresis, the gels were incubated for 15 min at room temperature with agitation in 0.1 M sodium-phosphate buffer, pH 6.2, containing 4 mM ascorbic acid and 4 mM H_2O_2. The gels were then washed with distilled water and stained with a solution of 0.125 M HCl containing 0.1% (w/v) potassium ferricyanide and 0.1% (w/v) ferric chloride. Ascorbate peroxidase was located as an achromatic band on a Prussian blue background, as a result of the reaction between ferric chloride and potassium ferrocyanide, the latter having been produced by the reduction of potassium ferricyanide with unreacted ascorbic acid.

3.3.4 Protein profiles

Short term responses to salt stress in salt tolerant and susceptible seedlings were also studied using the standard electrophoretic technique for proteins; SDS-PAGE.

3.3.4.1 Protein extraction

Plant tissue weighing about 200 mg was homogenized in 1ml of extraction buffer (50 mM Tris-HCl, pH7.4; 1 mM EDTA; 2 mM $MgCl_2$; 2 mM DTT; 2.5 mM PMSF, 0.1% Triton-X 100) in a pre-cooled mortar and pestle and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was collected and centrifuged again at 12,000 rpm for 10 min at 4°C. The clear supernatant was collected and used for protein quantification.

3.3.4.2 Protein estimation

Protein content was quantified by following Bradford's method for protein estimation (Bradford, 1976) using BSA (1mg/ml) as standard. The total protein content was expressed in mg/g dry wt.

3.3.4.3 SDS-PAGE

Discontinuous SDS-PAGE was performed with an electrophoresis unit (Bio-Rad) using 5% stacking gel and 12% resolving gel. An equal amount of total protein (50 μg) was mixed with 1XSDS gel-loading buffer and heated at 100 °C for 3 min to denature the protein. After electrophoresis, the gels were stained with Coomassie Brilliant Blue G-250 (Maniatis *et al.*, 1982).

3.3.5 Effect of GA_3 and $CaCl_2$

The effect of GA_3 and $CaCl_2$ on salinity stress was studied in two lines of pearl millet, ICMB 90111 and 841B–P3 (sensitive and highly tolerant, respectively, to NaCl stress) by germinating the seeds in presence of GA_3, $CaCl_2$ and a combination of both in Hoagland solution for the NaCl concentrations 0 mM, 75 mM, 100 mM and 150 mM. Calcium chloride ($CaCl_2$) and gibberellic acid (GA_3) at a concentration of 100 mg/l and 10 mg/l, respectively were added individually and in combination to Hoagland solution with NaCl concentrations 75mM, 100mM and 150mM, and the controls were maintained without any NaCl in Hoagland solution. The seedlings were allowed to grow for 15 days, harvested and washed with 0.5mM $CaCl_2$. They were scored for various parameters like germination efficiency, length of shoot/root, and proline content (Bates *et al.*, 1973). Fresh weights of shoot and root were measured for ten seedlings for each treatment and their mean was recorded.

3.4 Statistics

Experimental data were processed statistically using the GenStat software package (GenStat, 1995) and the levels of significance were ascertained for each source of variation in the ANOVA. A genotype × environment analysis was performed for the data sets using the same software to assess the significance of interactions between the 28 inbred lines and the 4 salinity treatments for each of the observed traits, across a set of 27 germinating entries for the 0 and 75 mM NaCl treatments (purple foliage and pericarp genotype IP18293–P152 did not germinate evenly due to dormancy, and so was not included in the statistical analyses), and subsets of 20 and 5 germinating entries for the 0, 75, and 100 mM NaCl, and 0, 75, 100, and 150 mM NaCl treatments, respectively.

3.5 Development of Molecular Markers for Salt Stress Tolerance

The novel marker technique for plant genotyping known as Target Region Amplification Polymorphism (TRAP), which uses two primers of 18 nucleotides to generate markers (Hu and Vick, 2003), was used for generating markers for salt stress in pearl millet. The work was carried out at M.S. Swaminathan Applied Genomics Laboratory (AGL), ICRISAT, Patancheru, India.

3.5.1 Plant material

Two inbred lines out of the 28 inbred lines screened earlier, *viz.*, Tift $23D_2B_1$-P1-P5 (salt tolerant) and WSIL-P8 (salt sensitive) were parents of a previously developed F_2-derived F_4 mapping population of 97 progenies available at ICRISAT- Patancheru.

3.5.2 Data mining for the DNA sequences of target genes

Three genes related to salinity stress were taken into consideration for developing molecular markers, *viz.* superoxide dismutase (SOD) gene, glutathione reductase (GR) gene and Δ^1-pyrroline 5-carboxylate synthetase (P5CS) gene.

- The EST sequences of the SOD, GR and P5CS genes were retrieved from the National Centre for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov) by giving the key words or the accession numbers of the sequences available in the literature. The EST sequences for all the three genes were obtained in FASTA format.

- A GRAMENE BLAST (www.gramene.org/multi/blastview) search was performed using the maize EST sequences of the concerned target genes (8 for SOD, 1 for P5CS, and 17 for GR) as 'query' against each of the cereal crops (barley, pearl millet, sorghum and rice) as 'subject'. One sequence from each set was selected based on the E value (preferred $>10^{-5}$), score (highest), % identity (<80%) and alignment length (highest). The 'maize-HSPs' (High-scoring Segment Pairs) obtained during BLAST searches for each crop for the concerned EST sequences were aligned (sequence

alignment) to identify the unique conserved sequences using CLUSTAL W (a multiple alignment program: http://www.ebi.ac.uk/clustalw).

- The identical portions of the aligned consensus sequences were selected as the most conserved regions across the species and these portions were picked from maize-HSPs also present in pearl millet to get the trimmed target sequence, which was used for the fixed primer design. The trimmed target sequence was pasted in the input window of the web-based 'ORF finder' of NCBI database (www.ncbi.nlm.nih.gov/gorf/gorf.html) and the open reading frame (ORF) was found. The ORF helped to check if the primers designed fell in the interphase of two exons or not and the primers that fell in this interphase were rejected.

3.5.3 Primer design

3.5.3.1 Fixed / EST primers

Fixed primers are the primers targeting the gene of interest and are designed from the conserved portions of EST sequences of the targeted gene across species as trimmed earlier. These fixed primers were designed in the following way (Hu and Vick, 2003):

a) The identified trimmed target sequences were inserted into the input window of the web-based PCR primer-designing program (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) (Rozen and Skaletsky, 2000).
b) The primer optimum size, maximum size, and minimum size were set to 18 nucleotides.
c) The primer optimum melting temperature (Tm), maximum Tm, and minimum Tm were set to 53°C, 55°C, and 50°C, respectively.
d) One of the primer sequences picked by the program was used.

The list of fixed primers designed is given in Table 3.2. Both forward and reverse primers designed from the target EST sequence were used as fixed primers.

Table 3.2 List of fixed primers designed in this study

S.No	Primer Name	Gene/Target	Primer sequence	Tm (°C)	Size (nt)
1	**GR6F**	*Glutathione Reductase*	CCATTCCACCACTATCTG	54.04	18
2	**GR6R**	*Glutathione Reductase*	GTGGCTCCACATTTAACC	54.04	18
3	**GR8F**	*Glutathione Reductase*	TGGTGGGCACTATGACTA	54.04	18
4	**GR8R**	*Glutathione Reductase*	TGATGCACCATACACGAG	54.04	18
5	**GR13F**	*Glutathione Reductase*	ACTTCTGATGAGGCCTTG	54.04	18
6	**GR13R**	*Glutathione Reductase*	GAGATTTGTCCCTGGATG	54.04	18
7	**SOD2F**	*Superoxide Dismutase*	GTATCTCTGGCCTCAAGC	56.32	18
8	**SOD2R**	*Superoxide Dismutase*	GGTCCAGCAAGAGGTATC	56.32	18
9	**SOD3F**	*Superoxide Dismutase*	GCCAGATCCCACTGA	50.67	15
10	**SOD3R**	*Superoxide Dismutase*	TCCGATGATCCCACA	47.94	15
11	**SOD5MF**	*Superoxide Dismutase*	GAAATGTGACAGCTGGAG	54.04	18
12	**SOD5MR**	*Superoxide Dismutase*	CAAGATCATCGGGATCAG	54.04	18
13	**SOD5SF**	*Superoxide Dismutase*	TGTCAACTGGACCACACT	54.04	18
14	**SOD5SR**	*Superoxide Dismutase*	CGTGAACAACAACAGCTC	54.04	18
15	**SOD6MF**	*Superoxide Dismutase*	GCAGAGCTGTTGTTGTTC	54.04	18
16	**SOD6MR**	*Superoxide Dismutase*	GAATGTTCAGGCTCGTCT	54.04	18
17	**SOD7F**	*Superoxide Dismutase*	TGCCGATTTTGTTCG	45.21	15
18	**SOD7R**	*Superoxide Dismutase*	GCAAACATCGGAAGC	47.94	15
19	**P5CSF**	*Δ^1-pyrroline 5-carboxylate synthetase*	CTGTGGCAAGTTCTCTGT	54.04	18
20	**P5CSR**	*Δ^1-pyrroline 5-carboxylate synthetase*	CACTGAATCTGGTGCTTG	54.04	18

3.5.3.2 Arbitrary primers

The arbitrary primers are primers having arbitrary sequence with either an AT- or GC-rich core to anneal with an intron or exon, respectively. Three principles were followed in the construction of each random primer as suggested by Li and Quiros (2001) and Hu and Vick (2003):

a) the selective nucleotides, 3–4 at the 3' end;
b) the core, 4–6 nucleotides with AT- or GC-rich regions; and
c) the filler sequences make the 5' end.

The general principles of PCR primer design were followed such as, avoidance of self-complementarity, maintenance of GC content (40% to 60%) for proper melting temperature of primers and retention of their correct internal stability. The list of arbitrary primers designed is given in Table 3.3.

3.5.4 Genomic DNA extraction

3.5.4.1 DNA extraction and purification

Around 25–30 seeds of each of the 97 $F_{2:4}$ self-bulk progenies of the mapping population and its two parents were sown in pots in the glasshouse. Bulk DNA was obtained from each of the 99 entries using a modified CTAB method (Mace *et al.,* 2003). DNA was further purified by Rnase digestion followed by extraction with phenol/chloroform/iso-amylalcohol (25:24:1) and ethanol precipitation as described by Mace *et al.* (2003). A few of the steps have been illustrated in Fig. 3.1.

Detailed description of the 96 well plate mini DNA extraction

A. <u>Preparation</u>

1. Steel balls (4 mm in size and 2 number per extraction tube), pre-chilled at –20°C for about 30 minutes, were added to the 12 × 8-well strip extraction tubes with strip caps (Marsh Biomarket, USA), which were kept on ice.
2. 3% CTAB buffer (3% w/v CTAB, 1.4 M NaCl, 20mM EDTA, 100mM Tris-HCl, pH 8.0, 0.17% β-mercaptoethonol) was pre-heated at 65°C in a water bath

Table 3.3 List of arbitrary primers designed in this study

S.No.	Primer Name	Primer sequence	Tm (°C)	Size (nt)
1	**TRAP_Ar01**	TGAGTCCAAACCGGATGC	56.32	18
2	**TRAP_Ar02**	TGAGTCCAAACCGGAGCT	56.32	18
3	**TRAP_Ar03**	TGAGTCCAAACCGGTCAG	56.32	18
4	**TRAP_Ar04**	GACTGCGTACGAATTTGAC	54.93	19
5	**TRAP_Ar05**	GACTGCGTACGAATTGACT	54.93	19
6	**TRAP_Ar06**	GGAACCAAACACATGCTGA	54.93	19
7	**TRAP_Ar07**	TTCTTCTTCCCTGGACCATG	57.78	20
8	**TRAP_Ar08**	CTATCTCTCGGGACCGTCA	59.25	19
9	**TRAP_Ar09**	TTCTTCTTCCCTGGACCGTA	57.78	20
10	**TRAP_Ar10**	GGAACCAAACACATGGCTT	54.93	19
11	**TRAP_Ar11**	CTATCTCTCGGGACCCGAA	59.25	19
12	**TRAP_Ar12**	GGAACCAAACACATGAAGA	52.77	19
13	**TRAP_Ar13**	TCATCTCAAACCATATACAC	51.63	20
14	**TRAP_Ar14**	TTCTTCTTCCCTGGACACTT	55.73	20
15	**TRAP_Ar15**	CTATCTCTCGGGACCAAAC	57.09	19

(Precision Scientific, model: Shaking water bath 50) before the start of the sample collection.

3. Leaf strips 15 cm long were collected from 20 randomly selected one-week-old seedlings (final weight approximately 30mg) for each genotype and cut in to small pieces (1 mm in length). These pieces were transferred to the extraction tubes, which were fitted in a box.

B. <u>Grinding and extraction</u>

1. 450 µl of pre-heated 3% CTAB buffer was added to each extraction tube containing leaf samples.
2. Grinding was carried out using a Sigma Geno/Grinder (Spex CertiPrep, USA) at 500 strokes/min for 5 min.
3. Grinding was repeated until the color of the solution became pale green and leaf strips were sufficiently macerated. After the first round of grinding, the tube boxes were taken out from the Geno/Grinder to be checked for leakage and were shaken to ensure proper mixing of leaf tissues with the extraction buffer.
4. After grinding, the box with the tubes was fixed in a locking device and incubated at 65°C in a water bath for 10 min with occasional manual shaking.

C. <u>Solvent extraction</u>

1. 450 µl of a chloroform : iso-amyl alcohol (24:1) mixture was added to each tube, mixed by inverting the tubes carefully and the samples were centrifuged at 6200 rpm for 10 min (Sigma laboratory centrifuge model 4K15C with QIAGEN rotor model NR09100: 2 × 1120 g SW).
2. After centrifugation the aqueous layer (approximately 300µl) was transferred to a fresh tube (Marsh Biomarket).

D. <u>Initial DNA precipitation</u>

1. To each tube containing aqueous layer, 0.7 volume (approximately 210µl) of cold (kept at -20°C) isopropanol was added, the solution was carefully mixed and the tubes were kept at -20°C for 10 min.

2. The samples were centrifuged (Sigma laboratory centrifuge model 4K15C with QIAGEN rotor model NR09100: 2 × 1120 g SW) at 6200rpm for 15 min.
3. The supernatant was decanted under a fume–hood and pellets were allowed to air dry (approximately 30 min.).

E. Rnase treatment

1. In order to remove co-isolated RNA, pellets were dissolved into 200μl of low salt TE buffer and 30μg of Rnase (stock 10 mg/ml).
2. The solution was mixed properly and incubated at 37°C for 30 min or overnight at room temperature.

F. Solvent extraction

1. After incubation, 200 μl of phenol:chloroform:isoamylalcohol (25:24:1) was carefully added to each tube, mixed and centrifuged (Sigma laboratory centrifuge model 4K15C with QIAGEN rotor model NR09100: 2 × 1120 g SW) at 5000 rpm for 10 min.
2. The aqueous layer in each tube was transferred to a fresh tube (Marsh Biomarket) and 200 μl of chloroform:isoamylalcohol (24:1) was added to each tube, mixed and centrifuged at 5000 rpm for 10 min (Sigma laboratory centrifuge model 4K15C with QIAGEN rotor model NR09100: 2 × 1120 g SW). The aqueous layer was transferred to a fresh tube (Marsh Biomarket).

G. DNA precipitation

1. 15μl (approximately 1/10th volume) 3 M sodium acetate (pH 5.2) and 300 μl (2 volumes) 100% ethanol (kept at -20°C) was added to each of the tubes and the mixture was subsequently incubated in a freezer (-20°C) for 5 min.
2. Following incubation at -20°C, the tubes were centrifuged (Sigma laboratory centrifuge model 4K15C with QIAGEN rotor model NR09100: 2 × 1120 g SW) at 6200 rpm for 15 min.

H. Ethanol wash

1. After centrifugation the supernatant was carefully decanted in order to ensure that the pellet remains inside the tube. To the tubes, 200 μl of 70% ethanol was added followed by centrifugation (Sigma laboratory centrifuge

model 4K15C with QIAGEN rotor model NR09100: 2 × 1120 g SW) at 5000 rpm for 5 min.

I. Final re-suspension

1. The supernatant was carefully decanted and the pellet was allowed to air dry for one hour.
2. Completely dried pellets were re-suspended in 100 µl of $T_{10}E_1$ buffer and kept overnight at room temperature to dissolve completely.
3. DNA samples were stored in 4°C.

3.5.4.2 Determination of quantity and quality of isolated DNA

The DNA quality was checked using 0.5% agarose gels stained with ethidium bromide (1mg/ml). One µl of DNA solution was mixed with 1µl of loading buffer for non-denaturing PAGE (5X) and 8µl of distilled water and loaded into a well in a 0.5% agarose gel. Standard DNA of 5 ng, 10 ng, 15 ng and 20 ng were also loaded in each well-row as a reference. The gel was run for 10 min after which the quality was checked under UV illumination. A smear of DNA indicated poor quality whereas a clear band indicated good quality. Samples of poor quality were re-extracted. The DNA quantity was assessed using a DNA plate reader (Spectrafluor Plus, Tecan, Switzerland). The DNA concentrations were normalized at 5 ng/µl using robotics [TECAN, Genesis Workstation 200] (Fig. 3.2) for PCR reactions.

The optimal fixed and arbitrary primer concentrations were predetermined following a 3-grid optimization protocol. PCR reactions were conducted in 96-well plates in a GeneAmp PCR system 9700® (PE-Applied Biosystems). The reactions were performed in volumes of 10µl with final concentrations of 5 ng template DNA, 0.6 p.m/µl fixed primer, 0.2 p.m/µl arbitrary primer, 2mM $MgCl_2$, 0.12mM dNTPs; 1X buffer and 0.2 U *Taq* polymerase (Bioline). The PCR was performed by initially denaturing the template DNA at 94°C for 5 min, followed by five cycles at 94°C for 45 s, 35°C for 45 s, and 72°C for 1 min, then by 35 cycles at 94°C for 45 s, 50°C for 45 s and 72°C for 1 min, and a final extension step of 72°C for 7 min (Hu and Vick, 2003).

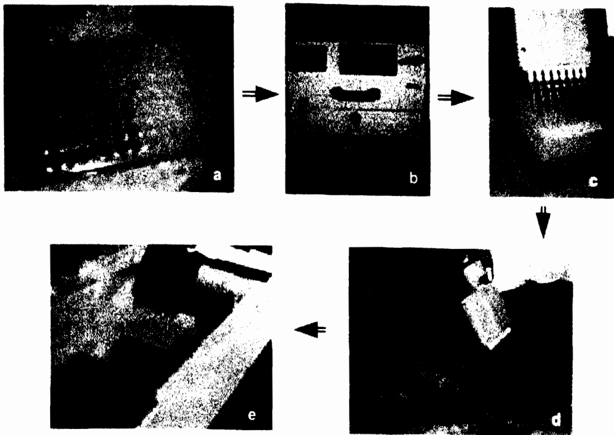


Figure 3.1. 96–well plate high throughput DNA extraction. Steps as per order : (a) Leaf sample collection → (b) Grinding in Sigma Geno/Grinder → (c) Solvent extraction → (d) Separation of aqueous phase → (e) DNA precipitation. (Photograph Source: ICRISAT, Patancheru)

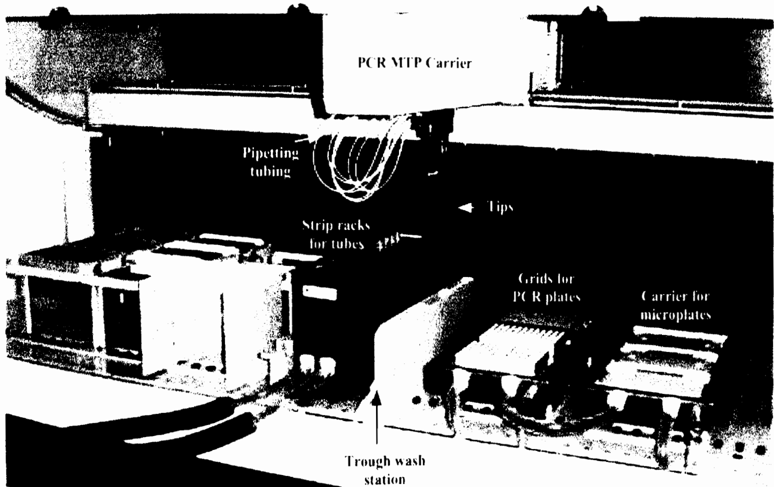


Figure 3.2 Robotics for 96–well DNA dilutions [**TECAN, Genesis Workstation 200**].
(Photograph Source: ICRISAT, Patancheru)

3.5.6 Testing parental polymorphism using TRAP markers

To identify TRAP markers detecting polymorphism between the mapping population parents, initial screening of the parental lines (Tift $23D_2B_1$-P1-P5 and WSIL-P8) was carried out before actual genotyping of all 97 progenies in the F_2-derived F_4 mapping populations. For parental polymorphism screening, PCR with parental DNA was performed using combinations of each fixed primer with all of the 15 arbitrary primers. The primer combinations producing polymorphic fragments were repeated a second time to test the reproducibility. Out of 180 fixed-arbitrary primer combinations tested, 11 were selected based on the highest number of clear polymorphic bands among the parents, to allow reliable genotyping of the mapping population progenies using PAGE. These 11 primer combinations were used to generate TRAP markers across the (Tift $23D_2B_1$-P1-P5 × WSIL-P8)-based pearl millet mapping population progeny set.

3.5.7 Poly-Acrylamide Gel Electrophoresis (PAGE)

3.5.7.1 Material

Sequencing gel apparatus (glass plates, spacers, casting apparatus), Combs (68-well) and D.C. power unit (Bio-Rad).

3.5.7.2 PAGE gel preparation

1. For a 7.7% gel (plate size 38 × 30 cm), 75 ml of gel solution was prepared as follows:

- 52.5 ml double distilled water
- 7.5 ml 10X TBE buffer
- 15.0 ml acrylamide solution (40% of acrylamide and 2% bis-acrylamide in ratio of 29:1 v/v)

2. The solution was mixed in a 200 ml Erlenmeyer flask, and 450 µl 10% (w/v) APS was added, followed by 100µl TEMED. The solution was mixed, then poured using a syringe (100 ml), that feeds between the glass plates, and the

comb inserted (upside down, in order to form wells in the gel). The acrylamide solution was then allowed to polymerize for 30–45 min.

Note 1: Gels can be stored overnight as long as the plate ends are wrapped in pre-wetted tissue paper (1X TBE) and covered with plastic film.

Note 2: Polymerization of the acrylamide/bisacrylamide is catalyzed by the addition of APS, so one has to be quick while pouring the solution between the plates. A way to check the polymerization of the solution is to leave a little acrylamide solution in the flask and check after some time whether the solution has solidified.

3. After polymerization, the gel was set up with the unit for electrophoresis. The lower tank, the back-plate and upper reservoir were filled with 0.5X TBE (approximately 250–300 ml, 200 ml and 400 ml respectively). The wells were cleaned by aspirating and dispensing TBE buffer in each well using a Pasteur pipette to remove small fragments of gel and tiny bubbles on top of the well and the comb was inserted on top of the well so that the comb tips just rest on the well (<1 mm deep).
4. The gel was pre-run for at least 10 min at 5 V/cm (600 V, 9 W).
5. To each PCR product, 1 µl of orange loading dye (10 ml 0.5M EDTA, 1 ml 5M NaCl, 50 ml glycerol, 39 ml distilled water and orange dye powder) was added for every 5 µl of product. From this mixture, 4 µl was loaded into a well of the PAGE gel (7.7%).
6. Along with the samples, 2µl of 100bp ladder (50ng/µl, Qiagen) was also loaded in the first and last lane of the gel to ensure proper sizing of amplified PCR fragments.
7. The gel was run at 600–650 V in 0.5X TBE buffer for 3 to 3.5 hours using a Bio-Rad sequencing gel apparatus.
8. After the run, the plates were carefully pulled apart, so that the gel remained attached to the front plate. PCR product banding patterns on the PAGE gels were visualized using silver staining.

3.5.7.3 Silver staining

After running the PAGE gel. DNA fragments separated were visualized using a modified Tegelstrom (1992) silver staining procedure.

In this silver staining procedure, the 'PAGE gel was kept in the following solutions with continuous shaking:

- 1.5 L of water for 5 min.
- 0.1% CTAB solution for 20 min (1.5 g in 1.5 L of water).
- 0.3% ammonia solution for 15 min (19.5ml of 25% ammonia in 1.5 L of water).
- 0.1% silver nitrate solution for 15 min (1.5g of silver nitrate + 6ml of 1M NaOH in 1.5 L of distilled water and neutralized with ammonia solution till the solution became colorless).
- 1.5 L of water for 15 s.
- Developer solution (22.5 g of sodium carbonate + 400 μl of formaldehyde in 1.5 L of single distilled water) till clear products were visible.
- 1.5 L of water for 30 s to 1 min.
- Fixer (22.5 ml glycerol in 1.5 L of water) for a few min.

3.5.8 Marker Data analysis

After silver staining of the PAGE gels, gels were put on a benchviewer. The size (in base pairs) of the parental alleles for each TRAP marker was estimated based on their migration relative to the 100bp DNA ladder (fragments ranging from 100bp to 1000bp) and presence or absence of only strong and unambiguous polymorphic bands in each parent as well as across the mapping population was manually scored. The $F_{2:4}$ progenies were scored as A and C or B and D for each polymorphic fragments based on presence or absence of bands of the parent lines, where,

B = WSIL-P8 homozygote (*i.e.*, band of Tift $23D_2B_1$-P1-P5 parent not present);

A = Tift $23D_2B_1$-P1-P5 homozygote (*i.e.*, band of WSIL-P8 parent not present);

C = Heterozygote or WSIL–P8 homozygote (band of WSIL-P8 parent present) *i.e.* not A; and

D = Heterozygote or Tift $23D_2B_1$-P1-P5 homozygote (band of Tift $23D_2B_1$-P1-P5 parent present), *i.e.* not B

- = Missing data for the individual at that locus.

After scoring each of the individual progenies, a dataset was assembled a Microsoft Excel spreadsheet in a format suitable for linkage analysis by Mapmaker/Exp. (*i.e.* rows = genotype score at a given locus; columns = individuals of mapping population).

3.5.9 Construction of genetic linkage map

The linkage map was constructed with Mapmaker/Exp 3.0 (Lander *et al*, 1987; Lincoln *et al.*, 1992a) on a personal computer. The segregation data for the TRAP markers were subjected to multi–point linkage analysis along with data for RFLP markers, which were generated previously for the Tift $23D_2B_1$-P1-P5 × WSIL-P8 based pearl millet mapping population (Liu *et al.*, 1994a,b). The LOD threshold value was kept at 3.0. Linkage distances in centimorgan (cM) units were calculated using the Kosambi mapping function (Kosambi 1944).

The 'sequence all' command was used for 'two-point' (or pair wise) linkage analysis of the data set, while the 'group' command was used to divide the markers into linkage groups. The 'compare' command was used to compute the maximum likelihood map for each specified order of markers, and obtain the orders sorted by likelihoods of their map. Mapmaker reports only the 20 most likely orders. The order having a *log-likelihood* of 0.0 was selected as the best order. The 'build' command was used to place new markers from the genotyping dataset at the most appropriate positions within the identified linkage group. The RFLP marker data used in this study have previously been mapped in this (Tift $23D_2B_1$–P1–P5 × WSIL–P8)-based pearl millet mapping population (Liu *et al.*, 1994a,b). The RFLP-based skeleton linkage map available for this mapping population was used as a reference for the map constructed in the current study for comparison with respect to linkage distances, linkage position and marker order of the RFLP markers. The TRAP markers used for the present study were thereby assigned to linkage groups based on their order with respect to the RFLP markers previously mapped in this (Tift $23D_2B_1$–P1–P5 × WSIL–P8)-based mapping population.

3.6 QTL analysis

3.6.1 Phenotypic data

Seeds of the parents and individuals of the $F_{2:4}$ mapping populations were surface sterilized and germinated at 20°C on filter paper boats in balanced nutrient solutions (Hoagland and Arnon, 1938) of pH 6.7, containing two different concentrations of NaCl (0mM and 150mM) in duplicates for each experiment and the seedlings were allowed to grow for 10 days at 25°C under continuous light. Salinity stress-related traits like germination efficiency (%) and length (cm) of shoot and root after 10 days of seedling growth were recorded for each experiment. Relative values as compared to the controls and deviations from the means were also calculated for germination efficiency, and for shoot and root lengths for each treatment.

3.6.2 Analysis of variance (ANOVA)

The analyses of variance for phenotypic data sets were performed using the residual maximum likelihood algorithm (ReML), which provided the best linear unbiased predictions (BLUPs) of the performance of the progenies (Patterson and Thompson, 1971). ReML estimates the components of variance by maximizing the likelihood of all contrasts with zero expectation. For each trait and for each entry, the predicted means were calculated with entries as fixed effects for both individual environment (salinity level) analyses and the across-environment (salinity levels) analyses; replications, error and entry × replication interactions as random effects in individual screening environment analyses; and replication, error, entry × replication, and entry × environment interactions as random effects in the across-salinity screening environment analysis.
Experimental data were analyzed statistically (Genstat 1995) to ascertain the levels of significance for each source of variation (replicates, genotypes, salinity levels and error in the experiment).

3.6.3 QTL mapping

A total of 122 (54 RFLP and 68 TRAP) markers were used to detect QTLs associated with germination and length of shoot and root under control and salt stressed conditions in pearl millet. Simple Interval Mapping (SIM) was

performed using MapMaker/QTL version 1.1b (Lincoln *et al.*, 1992b). The LOD threshold value was kept at 3.0. Composite Interval Mapping (CIM) was performed using PlabQTL version 1.2 (Utz and Melchinger, 2003), which employs interval mapping using a regression approach (Haley and Knott, 1992) with selected markers as cofactors. The point at which the LOD score had the maximum value in the interval was taken as the estimate of the QTL position. The proportion of phenotypic variance explained by a single QTL was estimated as the square of the partial correlation coefficient. Estimates of the additive effect of each detected QTL, the total LOD score, and the total proportion of phenotypic variance explained jointly by all detected QTL were obtained by fitting a multiple linear regression model that simultaneously included all detected QTL for the trait in question.

The percentage of phenotypic variance explained by a putative QTL (R^2%) was calculated, which is based on the partial correlation of the putative QTL with the observed variable, adjusted for cofactors (Kendall and Stuart, 1961). In the simultaneous fit, the cofactors are ignored and only the putative QTLs initially detected and their estimated positions were used in multiple regressions to obtain the final estimate of the additive effects and percentage of phenotypic variation for a particular trait that could be explained by the QTL(s). The adjusted R^2% (adjR^2%), the portion of the phenotypic variance explained by the final full model, was estimated according to Hospital *et al.* (1997). The additive effect was calculated as half the differences between genotypic values of the two homozygotes (Falconer, 1989):
Additive effect = (Parent P2 – Parent P1)/2.

After the QTL analysis with PlabQTL, the QTLs identified for components of salinity stress were assigned to the linkage groups based on linkage positions of markers on the skeleton-linkage map developed earlier.

RESULTS

CHAPTER 4

RESULTS

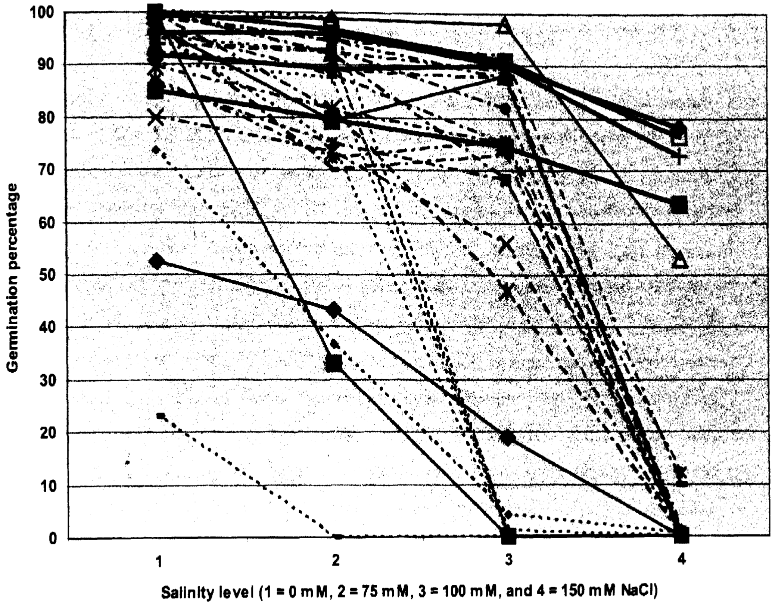
4.1 Screening and selection of pearl millet germplasm for salinity tolerance

The objective of this part of the investigation was to assess the exploitable genetic variability in pearl millet for salinity tolerance and to detect the salt-sensitive and salt-tolerant pearl millet lines from the germplasm screened. Twenty-eight genetically diverse inbred pearl millet genotypes (ICMP 85410-P7, LGD 1-B-10, Tift $23D_2B_1$-P1-P5, WSIL-P8, 81B-P6, ICMP 451-P8, ICMP 451-P6, H 77/833-2-P5(NT), H 77/833-2, PRLT 2/89-33, W 504-1-P1, P310-17-Bk, PT 732B-P2, P1449-2-P1, ICMB 841(=841B)-P3, 863B-P2, IP 18293-P152, Tift $238D_1$-P158, Tift 186, Tift 383, ICMB 89111, ICMB 90111, ICMB 92666, ICMB 95333, 843B, ICMB 98004, ICMB 99022, and ICML 22,) obtained from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India were used in this study.

4.1.1 Morphological parameters

Morphological symptoms are indications of the injurious effects of salt stress. The extent of inhibitory or adverse effects of salt stress can be known only by making critical comparisons with plants grown under comparable conditions. Compared to non-saline conditions, germination was significantly reduced at 75mM, 100mM and 150mM NaCl concentrations (Fig. 4.1). However, there were significant differences among the 28 genotypes in this respect. In most of the pearl millet inbred lines tested (except for IP 18293-P152, which showed a high level of seed dormancy), germination at 75mM NaCl was above 70% of their respective non-saline controls, indicating that the germination process in pearl millet was fairly tolerant to this level of salinity. However, at 100mM NaCl, seven of the 28 lines failed to germinate, and at 150mM NaCl the number of successfully germinated lines dropped to only five. There was considerable variation among genotypes in their germination percentage and survival at the two highest salinity levels. At 150mM NaCl, the survival percentages of only five

Figure 4.1. Changes in germination percentage of seeds of 28 pearl millet inbred lines across four salinity levels: [(1) 0mM NaCl (control), (2) 75mM NaCl, (3) 100mM NaCl, and (4) 150mM NaCl]



ICMP 85410-P7
81B-P6
P1449-2-P1
Tift 238D1-P152
ICMP 451-P6
H 77/833-2
W 504-1-P1
863B-P2
Tift 186
ICMB 89111 (tester)
843B
ICMB 99022 (843B-like)
P310-17-Bk
ICMB 95333 (tester)
WSIL-P8
PRLT 2/89-33
ICMB 90111 (tester)
IP 18293-P158 (dormant, purple)
ICMP 451-P8
H 77/833-2-P5(NT)
PT 732B-P2
LGD 1-B-10
Tift 383
ICMB 92666 (tester)
ICMB 98004 (843B-like)
Tift 23D2B1-P1-P5
ICMB 841-P3
ICML 22 (oasis germplasm selection)

accessions were good and there was over 80% mortality in most of the accessions at this salinity level. Salt burning symptoms, i.e. leaf yellowing and necrosis etc. started appearing from 15 days after germination. Hence all other morphological parameters were recorded after 10 days of seedling growth, before the seedlings were visibly injured by salt stress.

ANOVA (Table 4.1) clearly demonstrates the statistically significant contributions of the 28 inbred genotypes, the salinity level treatments (2 to 4), and the interactions between these (indicating the existence of genetic differences in salinity tolerance), to all ten pearl millet seedling traits observed in this study. Tabulated Residual Maximum Likelihood (ReML)-adjusted means for the germination percentages, root:shoot ratios of lengths, fresh and dry weights (Table 4.2, Table 4.3, Table 4.4 and Table 4.5) in all inbred × salinity level treatment combinations show that these statistically significant differences are in fact large enough to have biological meaning and potential economic importance. At the highest salt concentration, the maximum germination was recorded in Tift $23D_2B_1$-P1-P5 with 78.25% (Table 4.2). At salinity levels of 75mM and 100mM, the highest germination percentages were attained from the lines ICMP 85410-P7 and ICMB 95333, respectively. Among the five lines that grew till 150mM NaCl, Tift $23D_2B_1$-P1-P5 was affected the least by salinity because it gave the lowest percentage reduction for shoot length across the treatments (Figure 4.2). In general, shoot length diminished with increasing salinity levels in almost all cultivars. In many cases, pearl millet seedling root lengths were observed to increase with increased salt concentration [Figure 4.3]. Root length is one of the most important characters for salt stress because roots are in contact with soil and absorb water from soil. For this reason, root length provides an important clue to the response of plants to salt stress.

Similarly, when the fresh and dry weights [Figures 4.4, 4.5, 4.6 and 4.7] of salt treated shoots and roots were recorded and compared to those of controls, it was found that in many cases shoot and root fresh and dry weights remained unchanged or increased with increased salinity levels, at least for the first increment from 0 to 75 mM NaCl. Shoot length and dry matter production significantly decreased with increasing salinity (Figures 4.2 and 4.6). However,

Table 4.1 ANOVA mean sums of squares for ten characters in 28 pearl millet inbred lines screened *in vitro* for seed germination (all 28 entries) and early seedling growth (germinating entries only) across three salt concentrations (C2-C4) and a non-saline control (C1). C1 = Non-saline control = 0 mM NaCl; C2 = 75 mM NaCl; C3 =1 00 mM NaCl; C4 = 150 mM NaCl

Source	df (except for germination)			Germination (%)		Shoot Length (cm)			Root Length (cm)			Root:Shoot Ratio (Length Basis)		
	C1 & C2	C1; C2 & C3	C1, C2, C3 & C4	df	C1, C2, C3 & C4	C1 & C2	C1, C2 & C3	C1, C2, C3 & C4	C1 & C2	C1, C2 & C3	C1, C2, C3 & C4	C1 & C2	C1, C2 & C3	C1, C2, C3 & C4
Replication	3	3	3	3	6.80	0.33	0.24	1.22	0.84	0.74	1.57	0.005	0.005	0.055
Inbred	27	21	9	27	22194.04	31.95	26.58	46.20	43.62	56.77	67.46	0.641	0.756	1.542
Salinity level	1	2	3	1	8924.03	58.89	158.73	172.65	7.94	9.01	127.50	1.768	2.552	0.869
Inbred × salinity level	26(1)	42	27	26(1)	**1031.09**	**9.06**	**14.20**	**18.49**	**8.60**	**16.70**	**18.31**	**0.161**	**0.282**	**0.292**
Error	161 (4)	190 (5)	109 (8)	161(4)	3.52	0.18	0.20	0.53	0.18	0.24	0.53	0.003	0.005	0.035

Source	df			Shoot Fresh Weight (mg)			Root Fresh Weight (mg)			Root:Shoot Ratio (Fresh Weight Basis)		
	C1 & C2	C1, C2 & C3	C1, C2, C3 & C4	C1 & C2	C1, C2 & C3	C1, C2, C3 & C4	C1 & C2	C1, C2 & C3	C1, C2, C3 & C4	C1 & C2	C1, C2 & C3	C1, C2, C3 & C4
Replication	3	3	3	2.772	2.192	1.708	0.093	0.034	0.058	0.005	0.005	0.055
Inbred	27	21	9	2500.128	2467.625	2671.583	511.014	437.800	227.175	0.641	0.756	1.542
Salinity level	1	2	3	820.015	4198.647	5183.541	1.282	140.800	416.434	1.768	2.552	0.869
Inbred × salinity level	26(1)	42	27	**81.419**	**344.976**	**342.816**	**12.534**	**86.090**	**79.163**	**0.161**	**0.282**	**0.292**
Error	161 (4)	190 (5)	109 (8)	1.097	0.960	2.464	0.133	0.094	0.143	0.003	0.005	0.035

Table 4.1 contd.

Source	df			Shoot Dry Weight (mg)			Root Dry Weight (mg)			Root:Shoot Ratio (Dry Weight Basis)		
	C1 & C2	C1, C2 & C3	C1, C2, C3 & C4	C1 & C2	C1, C2 & C3	C1, C2, C3 & C4	C1 & C2	C1, C2 & C3	C1, C2, C3 & C4	C1 & C2	C1, C2 & C3	C1, C2, C3 & C4
Replication	3	3	3	0.026	0.031	0.011	0.005	0.009	0.025	0.005	0.005	0.055
Inbred	27	21	9	296.277	492.200	790.801	8.922	15.811	21.232	0.641	0.756	1.542
Salinity level	1	2	3	2.994	28.860	38.146	0.082	0.527	3.426	1.768	2.552	0.869
Inbred × salinity level	26(1)	42	27	**4.075**	**6.322**	**9.476**	**0.272**	**0.217**	**0.547**	**0.161**	**0.282**	**0.292**
Error	161 (4)	190 (5)	109 (8)	0.039	0.030	0.038	0.008	0.005	0.006	0.003	0.005	0.035

Table 4.2 ReML-adjusted entry means for 28 pearl millet inbreds screened *in vitro* in Hoagland's solution for germination in three treatments varying in NaCl concentration and in a non-saline control treatment

Entry No.	Entry Name	0 mM NaCl	75 mM NaCl	100 mM NaCl	150 mM NaCl
1	ICMP 85410-P7	100.00	99.25	0.00	0.00
2	WSIL-P8	95.25	92.50	0.00	0.00
3	81B-P6	100.00	92.00	1.25	0.00
4	PRLT 2/89-33	73.75	36.75	4.25	0.50
5	P1449-2-P1	95.75	75.25	0.00	0.00
6	ICMB 90111	100.00	33.00	0.00	0.00
7	Tift $238D_1$-P158	90.25	87.50	0.00	0.00
8	IP 18293-P152	23.25	0.00	0.00	0.00
9	ICMP 451-P6	99.75	81.25	75.25	0.00
10	ICMP 451-P8	92.50	89.00	87.50	0.00
11	H 77/833-2	100.00	92.00	68.25	0.75
12	H 77/833-2-P5(NT)	99.00	94.75	87.50	1.25
13	W 504-1-P1	86.25	75.25	68.00	0.00
14	PT 732B-P2	87.00	72.25	75.75	12.25
15	863B-P2	93.75	93.00	82.00	0.00
16	LGD 1-B-10	96.75	88.25	75.25	0.00
17	Tift 186	97.50	70.00	73.00	0.00
18	Tift 383	99.50	94.75	91.75	10.00
19	ICMB 89111	52.50	43.25	19.00	0.00
20	ICMB 92666	97.25	79.50	88.25	0.00
21	843B	93.75	92.25	89.00	0.00
22	ICMB 98004	80.00	73.50	56.00	0.00
23	ICMB 99022	89.50	81.75	47.00	0.00
24	Tift $23D_2B_1$-P1-P5	91.75	89.25	90.25	78.25
25	P310-17-Bk	96.00	96.00	90.00	72.75
26	841B-P3	100.00	97.00	91.00	76.50
27	ICMB 95333	99.25	98.75	98.00	53.00
28	ICML 22	85.00	79.75	74.50	63.50
	Salinity treatment grand mean	89.83	78.49	54.74	13.17
	SE (+/-)	1.69	4.71	1.30	1.19
	CV (%)	3.77	11.99	4.74	18.11
	F-ratio	**95.84**	**24.71**	**867.08**	**508.19**
	$h^{2,1}$	0.96	0.86	1.00	0.99
	$h^{2,2}$	0.99	0.96	1.00	1.00

$h^{2,1}$ = operational heritability calculated on the basis of entry mean values

$h^{2,2}$ = operational heritability calculated on the basis of plot values

Table 4.3 ReML-adjusted entry means for 28 pearl millet inbreds screened *in vitro* in Hoagland's solution for root/shoot ratio (based on lengths) in three treatments varying in NaCl concentration and in a non-saline control treatment

Entry No.	Entry Name	Root/Shoot Ratio (Length Basis)			
		0 mM NaCl	75 mM NaCl	100 mM NaCl	150 mM NaCl
1	ICMP 85410-P7	0.77	0.61	-	-
2	WSIL-P8	0.65	0.68	-	-
3	81B-P6	0.49	0.83	0.82	-
4	PRLT 2/89-33	0.51	0.52	0.50	0.30
5	P1449-2-P1	0.84	1.09	-	-
6	ICMB 90111	0.91	0.86	-	-
7	Tift $238D_1$-P158	0.59	0.60	-	-
8	IP 18293-P152	0.22	-	-	-
9	ICMP 451-P6	1.29	2.31	1.32	-
10	ICMP 451-P8	0.53	1.09	1.59	-
11	H 77/833-2	1.09	1.34	2.14	1.99
12	H 77/833-2-P5(NT)	1.06	1.22	1.01	1.73
13	W 504-1-P1	1.05	1.08	1.15	-
14	PT 732B-P2	1.02	0.92	1.06	1.77
15	863B-P2	0.84	0.61	1.15	-
16	LGD 1-B-10	0.70	0.74	0.45	-
17	Tift 186	1.04	1.14	1.02	-
18	Tift 383	0.84	1.17	1.37	1.13
19	ICMB 89111	1.09	1.20	1.05	-
20	ICMB 92666	0.87	0.99	1.52	-
21	843B	0.53	1.19	1.14	-
22	ICMB 98004	0.85	0.77	1.56	-
23	ICMB 99022	0.77	0.78	1.46	-
24	Tift $23D_2B_1$-P1-P5	1.05	1.49	1.19	0.88
25	P310-17-Bk	1.08	1.05	1.07	1.19
26	841B-P3	0.63	1.06	0.86	0.78
27	ICMB 95333	0.47	0.96	1.42	1.00
28	ICML 22	0.82	0.81	1.12	0.91
	Salinity treatment grand mean	0.81	1.00	1,18	1.17
	SE (+/-)	0.02	0.03	0.05	0.17
	CV (%)	5.18	6.01	8.18	29.12
	F-ratio	**143.59**	**140.46**	**58.78**	**9.36**
	$h^{2,1}$	0.97	0.97	0.94	0.68
	$h^{2,2}$	0.99	0.99	0.98	0.89

$h^{2,1}$ = operational heritability calculated on the basis of entry mean values

$h^{2,2}$ = operational heritability calculated on the basis of plot values

Table 4.4 ReML-adjusted entry means for 28 pearl millet inbreds screened *in vitro* in Hoagland's solution for root/shoot ratios (based on fresh weights) in three treatments varying in NaCl concentration and in a non-saline control treatment

Entry No.	Entry Name	Root/Shoot Ratio (Fresh Weight Basis)			
		0 mM NaCl	75 mM NaCl	100 mM NaCl	150 mM NaCl
1	ICMP 85410-P7	0.30	0.43	-	-
2	WSIL-P8	0.35	0.33	-	-
3	81B-P6	0.36	0.38	0.17	-
4	PRLT 2/89-33	0.38	0.48	0.22	0.24
5	P1449-2-P1	0.50	0.48	-	-
6	ICMB 90111	0.30	0.24	-	-
7	Tift $238D_1$-P158	0.57	0.79	-	-
8	IP 18293-P152	0.19	-	-	-
9	ICMP 451-P6	0.27	0.29	0.35	-
10	ICMP 451-P8	0.41	0.43	0.55	-
11	H 77/833-2	0.22	0.21	0.19	0.21
12	H 77/833-2-P5(NT)	0.26	0.23	0.23	0.29
13	W 504-1-P1	0.19	0.22	0.28	-
14	PT 732B-P2	0.17	0.19	0.29	0.28
15	863B-P2	0.24	0.28	0.33	-
16	LGD 1-B-10	0.21	0.23	0.29	-
17	Tift 186	0.23	0.25	0.29	-
18	Tift 383	0.31	0.32	0.36	0.25
19	ICMB 89111	0.32	0.36	0.48	-
20	ICMB 92666	0.14	0.19	0.23	-
21	843B	0.15	0.16	0.16	-
22	ICMB 98004	0.33	0.36	0.32	-
23	ICMB 99022	0.15	0.17	0.24	-
24	Tift $23D_2B_1$-P1-P5	0.24	0.25	0.30	0.28
25	P310-17-Bk	0.24	0.24	0.23	0.27
26	841B-P3	0.39	0.31	0.30	0.25
27	ICMB 95333	0.16	0.17	0.18	0.18
28	ICML 22	0.28	0.47	0.23	0.25
	Salinity treatment grand mean	0.28	0.31	0.28	0.25
	SE (+/-)	0.00	0.00	0.01	0.00
	CV (%)	2.69	2.94	4.47	3.24
	F-ratio	**777.34**	**909.35**	**223.83**	**80.58**
	$h^{2,1}$	0.99	1.00	0.98	0.95
	$h^{2,2}$	1.00	1.00	1.00	0.99

$h^{2,1}$ = operational heritability calculated on the basis of entry mean values

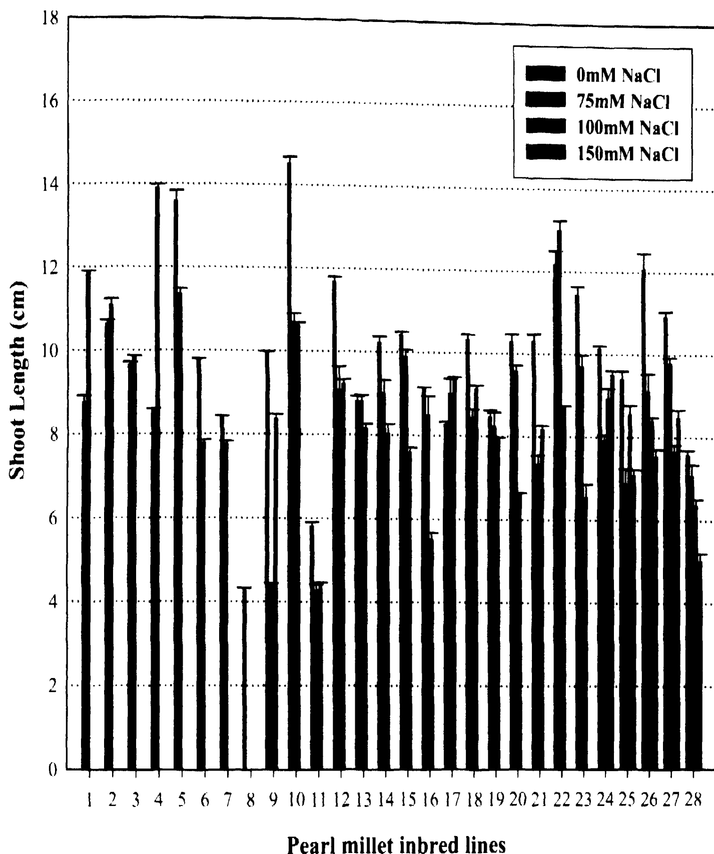
$h^{2,2}$ = operational heritability calculated on the basis of plot values

Table 4.5 ReML-adjusted entry means for 28 pearl millet inbreds screened *in vitro* in Hoagland's solution for shoot and root dry weights and root/shoot ratios (based on dry weights) in three treatments varying in NaCl concentrations and in a non-saline control treatment

Entry No.	Entry Name	Root/Shoot Ratio (Dry Weight Basis)			
		0 mM NaCl	75 mM NaCl	100 mM NaCl	150 mM NaCl
1	ICMP 85410-P7	0.14	0.24	-	-
2	WSIL-P8	0.32	0.35	-	-
3	81B-P6	0.26	0.21	0.00	-
4	PRLT 2/89-33	0.24	0.22	0.20	0.14
5	P1449-2-P1	0.32	0.18	-	-
6	ICMB 90111	0.52	0.24	-	-
7	Tift 238D1-P158	0.32	0.54	-	-
8	IP 18293-P152	0.52	-	-	-
9	ICMP 451-P6	0.73	0.74	0.90	-
10	ICMP 451-P8	0.24	0.32	0.34	-
11	H 77/833-2	0.35	0.35	0.29	0.42
12	H 77/833-2-P5(NT)	0.42	0.30	0.33	0.23
13	W 504-1-P1	0.40	0.36	0.23	-
14	PT 732B-P2	0.19	0.19	0.22	0.22
15	863B-P2	0.18	0.21	0.19	-
16	LGD 1-B-10	0.08	0.08	0.06	-
17	Tift 186	0.12	0.12	0.09	-
18	Tift 383	0.40	0.36	0.32	0.20
19	ICMB 89111	0.27	0.30	0.42	-
20	ICMB 92666	0.22	0.35	0.58	-
21	843B	0.07	0.07	0.08	-
22	ICMB 98004	0.30	0.25	0.20	-
23	ICMB 99022	0.19	0.20	0.19	-
24	Tift 23D2B1-P1-P5	0.13	0.14	0.16	0.12
25	P310-17-Bk	0.05	0.05	0.05	0.03
26	841B-P3	0.17	0.16	0.15	0.12
27	ICMB 95333	0.18	0.23	0.31	0.23
28	ICML 22	0.17	0.19	0.22	0.14
	Salinity treatment grand mean	0.27	0.26	0.25	0.18
	SE (+/-)	0.01	0.01	0.01	0.01
	CV (%)	11.09	7.53	7.74	13.97
	F-ratio	**108.07**	**224.61**	**373.65**	**71.98**
	$h^{2,1}$	0.96	0.98	0.99	0.95
	$h^{2,2}$	0.99	1.00	1.00	0.99

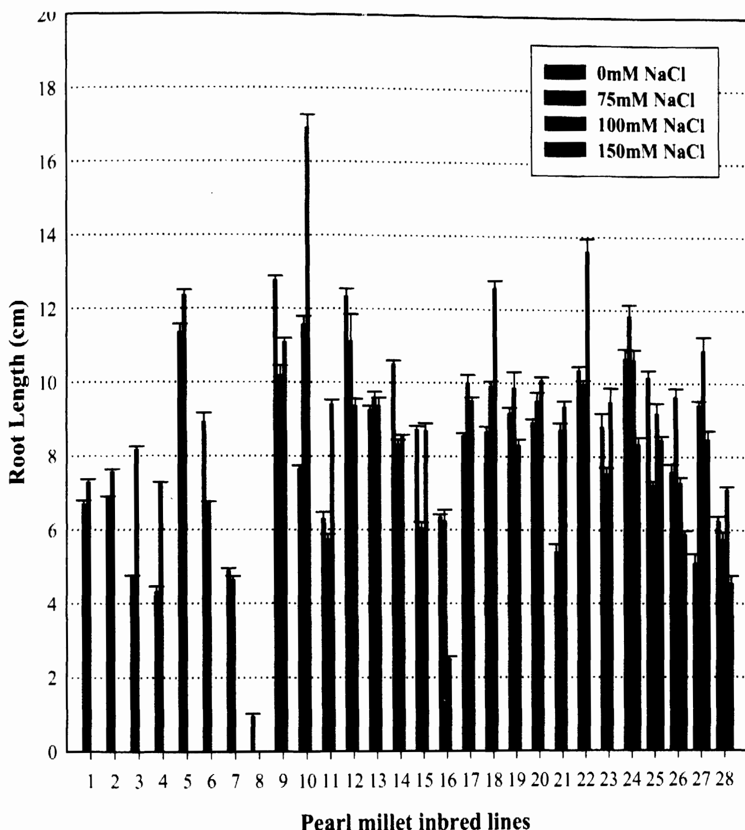
$h^{2,1}$ = operational heritablity calculated on the basis of entry mean values
$h^{2,2}$ = operational heritablity calculated on the basis of plot values

Figure 4.2. Changes in shoot lengths of 28 pearl millet inbred lines germinated and grown at four salinity levels



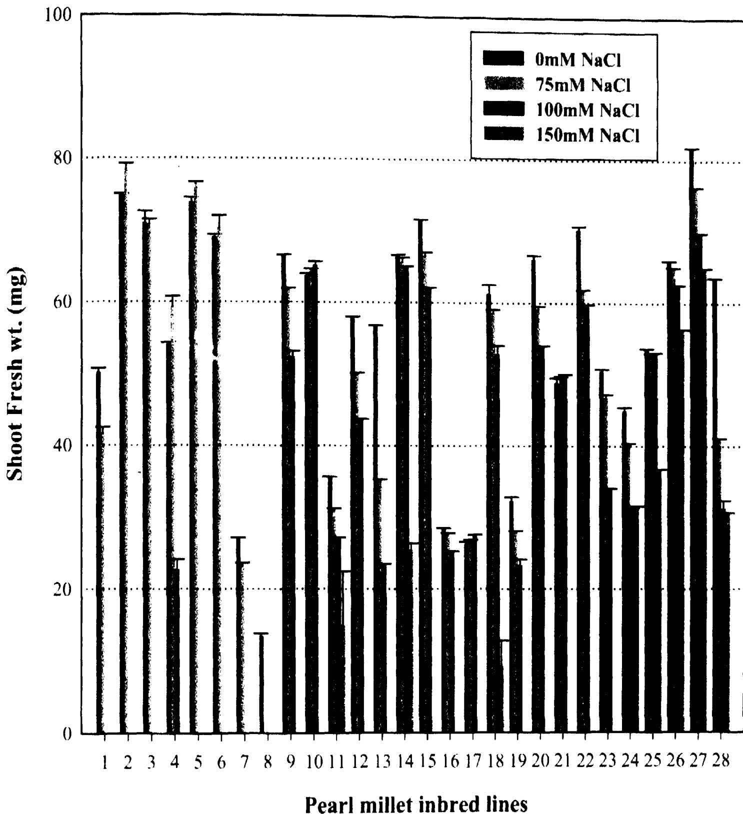
[Inbred lines: (1) ICMP 85410-P7, (2) WSIL-P8, (3) 81B-P6, (4) PRLT 2/89-33, (5) P1449-2-P1, (6) ICMB 90111, (7) Tift $238D_1$-P158, (8) IP 18293-P152, (9) ICMP 451-P6, (10) ICMP 451-P8, (11) H 77/833-2, (12) H 77/833-2-P5 (NT), (13) W 504-1-P1, (14) PT 732B-P2, (15) 863B-P2, (16) LGD 1-B-10, (17) Tift 186, (18) Tift 383, (19) ICMB 89111, (20) ICMB 92666, (21) 843B, (22) ICMB 98004, (23) ICMB 99022, (24) Tift $23D_2B_1$-P1-P5, (25) P310-17-Bk, (26) ICMB 841-P3, (27) ICMB 95333, (28) ICML 22]

Figure 4.3. Changes in root lengths of 28 pearl millet inbred lines germinated and grown at four salinity levels



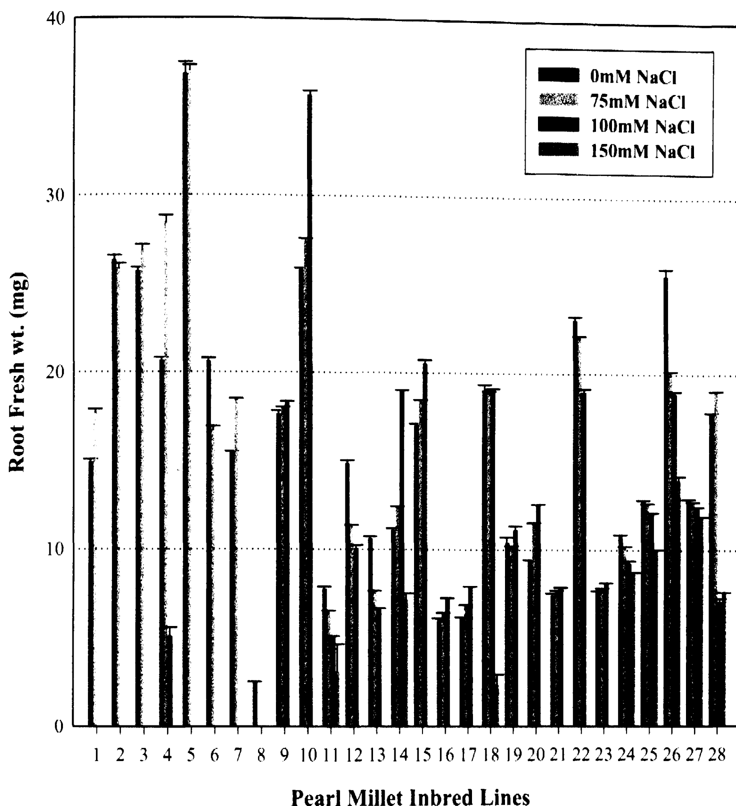
[Inbred lines: (1) ICMP 85410-P7, (2) WSIL-P8, (3) 81B-P6, (4) PRLT 2/89-33, (5) P1449-2-P1, (6) ICMB 90111, (7) Tift 238D$_1$-P158, (8) IP 18293-P152, (9) ICMP 451-P6, (10) ICMP 451-P8, (11) H 77/833-2, (12) H 77/833-2-P5 (NT), (13) W 504-1-P1, (14) PT 732B-P2, (15) 863B-P2, (16) LGD 1-B-10, (17) Tift 186, (18) Tift 383, (19) ICMB 89111, (20) ICMB 92666, (21) 843B, (22) ICMB 98004, (23) ICMB 99022, (24) Tift 23D$_2$B$_1$-P1-P5, (25) P310-17-Bk, (26) ICMB 841-P3, (27) ICMB 95333, (28) ICML 22]

Figure 4.4. Changes in shoot fresh weights of 28 pearl millet inbred lines germinated and grown at four salinity levels



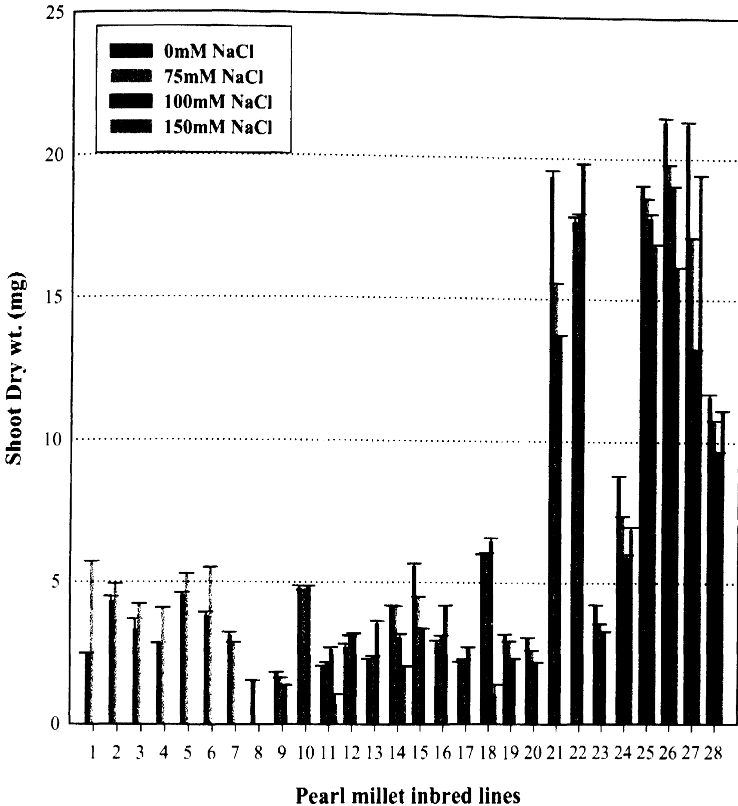
[Inbred lines: (1) ICMP 85410-P7, (2) WSIL-P8, (3) 81B-P6, (4) PRLT 2/89-33, (5) P1449-2-P1, (6) ICMB 90111, (7) Tift $238D_1$-P158, (8) IP 18293-P152, (9) ICMP 451-P6, (10) ICMP 451-P8, (11) H 77/833-2, (12) H 77/833-2-P5 (NT), (13) W 504-1-P1, (14) PT 732B-P2, (15) 863B-P2, (16) LGD 1-B-10, (17) Tift 186, (18) Tift 383, (19) ICMB 89111, (20) ICMB 92666, (21) 843B, (22) ICMB 98004, (23) ICMB 99022, (24) Tift $23D_2B_1$-P1-P5, (25) P310-17-Bk, (26) ICMB 841-P3, (27) ICMB 95333, (28) ICML 22]

Figure 4.5. Changes in root fresh weights of 28 pearl millet inbred lines germinated and grown at four salinity levels



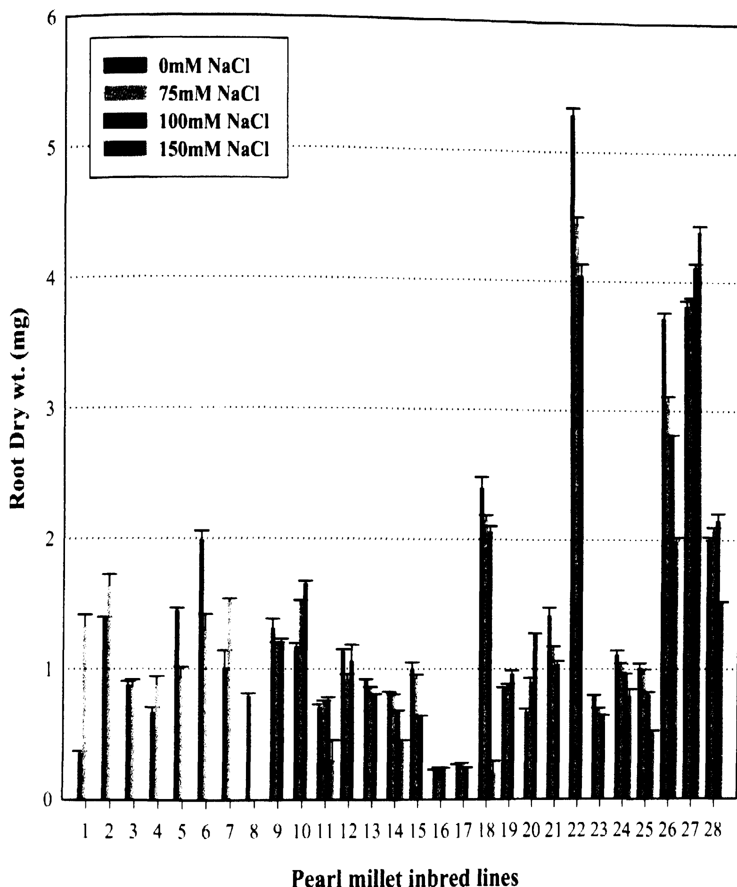
[Inbred lines: (1) ICMP 85410-P7, (2) WSIL-P8, (3) 81B-P6, (4) PRLT 2/89-33, (5) P1449-2-P1, (6) ICMB 90111, (7) Tift 238D$_1$-P158, (8) IP 18293-P152, (9) ICMP 451-P6, (10) ICMP 451-P8, (11) H 77/833-2, (12) H 77/833-2-P5 (NT), (13) W 504-1-P1, (14) PT 732B-P2, (15) 863B-P2, (16) LGD 1-B-10, (17) Tift 186, (18) Tift 383, (19) ICMB 89111, (20) ICMB 92666, (21) 843B, (22) ICMB 98004, (23) ICMB 99022, (24) Tift 23D$_2$B$_1$-P1-P5, (25) P310-17-Bk, (26) ICMB 841-P3, (27) ICMB 95333, (28) ICML 22]

Figure 4.6. Changes in shoot dry weights of 28 pearl millet inbred lines germinated and grown at four salinity levels



[Inbred lines: (1) ICMP 85410-P7, (2) WSIL-P8, (3) 81B-P6, (4) PRLT 2/89-33, (5) P1449-2-P1, (6) ICMB 90111, (7) Tift 238D$_1$-P158, (8) IP 18293-P152, (9) ICMP 451-P6, (10) ICMP 451-P8, (11) H 77/833-2, (12) H 77/833-2-P5 (NT), (13) W 504-1-P1, (14) PT 732B-P2, (15) 863B-P2, (16) LGD 1-B-10, (17) Tift 186, (18) Tift 383, (19) ICMB 89111, (20) ICMB 92666, (21) 843B, (22) ICMB 98004, (23) ICMB 99022, (24) Tift 23D$_2$B$_1$-P1-P5, (25) P310-17-Bk, (26) ICMB 841-P3, (27) ICMB 95333, (28) ICML 22]

Figure 4.7. Changes in root dry weights of 28 pearl millet inbred lines germinated and grown at four salinity levels



[Inbred lines: (1) ICMP 85410-P7, (2) WSIL-P8, (3) 81B-P6, (4) PRLT 2/89-33, (5) P1449-2-P1, (6) ICMB 90111, (7) Tift $238D_1$-P158, (8) IP 18293-P152, (9) ICMP 451-P6, (10) ICMP 451-P8, (11) H 77/833-2, (12) H 77/833-2-P5 (NT), (13) W 504-1-P1, (14) PT 732B-P2, (15) 863B-P2, (16) LGD 1-B-10, (17) Tift 186, (18) Tift 383, (19) ICMB 89111, (20) ICMB 92666, (21) 843B, (22) ICMB 98004, (23) ICMB 99022, (24) Tift $23D_2B_1$-P1-P5, (25) P310-17-Bk, (26) ICMB 841-P3, (27) ICMB 95333, (28) ICML 22]

the relative reduction of shoot length and shoot dry matter (shoot dry weight mg/seedling) at various salinity levels was less in the most tolerant lines compared to the least tolerant lines (Tables 4.6 and 4.7). There was large variation in salinity tolerance assessed as relative root dry weight. With an increase of NaCl concentrations in the growth solution, ICMB 95333 showed an exceptional increase in root dry weight with salinity, while the other four accessions germinating till 150mM NaCl showed a gradual reduction in dry weight of roots with increasing salinity levels. At 75mM NaCl, the differences between the most and the least tolerant lines were not clear, but at 100mM and 150mM NaCl, the differences in germination, shoot length and shoot dry weight between the most and least tolerant lines were significant. A sub-sample of six pearl millet accessions representing tolerant (Tift $23D_2B_1$-P1-P5 and 841B-P3), moderately tolerant (863B-P2 and 843B) and sensitive (WSIL-P8 and ICMB 90111) accessions were germinated and tested against five NaCl concentrations, and their responses for absolute root lengths are presented in Figure 4.8. Clearly, significant differences in the responses of these three representative classes of accessions to NaCl were observed.

Based on growth performance (survival/germination %, shoot/root lengths, and shoot/root dry matter production) the pearl millet accessions were grouped into three categories:

1. The first category includes accessions where the seeds germinated in the salt medium only up to 75mM NaCl concentration without any defect. Their germination in 100mM and 150mM NaCl media was only 0-5% and 0-0.6% of their respective controls (Table 4.7). Shoot dry matter production of these lines in 75mM NaCl ranged from 89.1% in Tift $238D_1$-P158 to 227.9% in ICMP 85410-P7 relative to their respective non-saline controls. The lines in this group were categorized as 'sensitive' to salt stress during germination and early seedling growth.
2. The second category includes accessions where seeds germinated without any defective symptoms in the salt media up to 100mM NaCl concentration. Their shoot dry weights in 75mM NaCl were in the range of 79.9% in 843B to 114.8% in H 77/833-2-P5 (NT) of their respective non-saline controls

Table 4.6 Relative shoot and root length (%) of 10-day old seedlings of 27 pearl millet accessions germinated and grown at four NaCl concentrations.

Accession	Relative Shoot Length (%)			Relative Root Length (%)		
	75mM	100mM	150mM	75mM	100mM	150mM
ICMP 85410-P7	135.23	-	-	108.96	-	-
WSIL-P8	103.74	-	-	110.14	-	-
81B-P6	102.06	6.19*	-	174.47	12.77*	-
PRLT 2/89-33	161.63	26.74*	4.65*	169.77	34.88*	-4.65*
P1449-2-P1	83.82	-	-	108.77	-	-
ICMB 90111	79.59	-	-	75.28	-	-
Tift 238D1-P158	92.86	-	-	95.92	-	-
ICMP 451-P6	44.00	-	-	79.69	-	-
ICMP 451-P8	73.79	73.10	-	150.65	219.48	-
H 77/833-2	74.14	75.86	-	90.48	149.21	17.46*
H 77/833-2-P5(NT)	73.50	78.63	23.93*	85.37	76.42	26.02*
W 504-1-P1	100.00	93.18	-	103.23	101.08	-
PT 732B-P2	88.24	79.41	41.18*	79.05	80.95	-
863B-P2	95.19	73.08	-	70.11	100.00	-
LGD 1-B-10	93.41	60.44	-	98.44	39.06	-
Tift 186	106.02	113.25	-	116.28	110.47	-
Tift 383	82.52	89.32	24.27*	113.79	144.83	21.84*
ICMB 89111	97.65	92.94	-	107.61	90.22	-
ICMB 92666	93.20	64.08	-	106.74	113.48	-
843B	70.87	79.61	-	161.11	174.07	-
ICMB 98004	106.56	71.31	-	96.15	130.77	-
ICMB 99022	84.35	57.39	-	85.23	107.95	-
Tift 23D2B1-P1-P5	78.22	88.12	94.06	111.21	100.00	78.50
P310-17-Bk	73.40	91.49	75.53	70.59	90.20	83.33
841B-P3	75.21	69.42	62.81	126.32	96.05	77.63
ICMB 95333	89.91	70.64	77.98	184.31	213.73	166.67
ICML 22	93.42	84.21	65.79	91.94	114.52	74.19
Mean	90.83	73.75	47.88	110.06	108.49	60.86
Std. Deviation	21.83	22.60	31.50	31.97	50.67	48.79
Std. Error	4.20	4.82	9.96	6.15	10.80	15.43

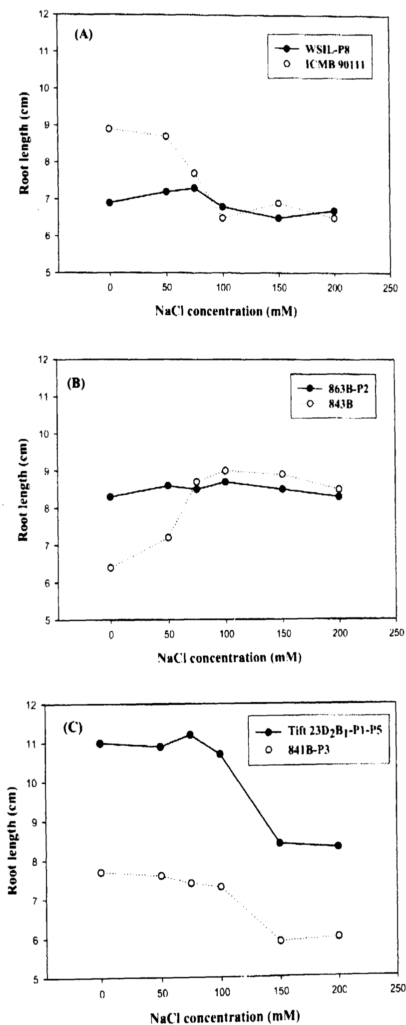
* Values not considered for salt tolerance comparisons as germination levels were negligible at that salt level.

Table 4.7 Relative shoot and root dry weight (%) of 10-day old seedlings of 27 pearl millet accessions germinated and grown at four NaCl concentrations.

Accession	Dry Shoot Weight (mg)			Dry Root Weight (mg)		
	75mM	100mM	150mM	75mM	100mM	150mM
ICMP 85410-P7	227.94	-	-	380.00	-	-
WSIL-P8	109.98	-	-	119.57	-	-
81B-P6	125.60	0.30*	-	100.00	0.00*	-
PRLT 2/89-33	145.36	241.43*	302.14*	132.84	200.00*	177.61*
P1449-2-P1	114.54	-	-	64.83	-	-
ICMB 90111	139.90	-	-	65.33	-	-
Tift 238D_1-P158	89.14	-	-	150.50	-	-
ICMP 451-P6	90.50	74.86	-	91.60	92.37	-
ICMP 451-P8	97.70	101.46	-	129.06	141.88	-
H 77/833-2	106.97	130.35	50.75*	107.14	107.14	61.43*
H 77/833-2-P5(NT)	114.81	117.78	64.44*	81.42	93.81	35.40*
W 504-1-P1	104.82	154.82	-	94.51	87.91	-
PT 732B-P2	98.81	72.55	-	100.00	83.75	-
863B-P2	79.93	60.04	-	93.00	63.00	-
LGD 1-B-10	107.67	145.64	-	109.09	104.55	-
Tift 186	104.15	125.35	-	103.85	92.31	-
Tift 383	101.01	108.56	17.11*	89.96	86.19	8.37*
ICMB 89111	91.46	72.47	-	103.57	114.29	-
ICMB 92666	85.05	72.09	-	134.33	188.06	-
843B	79.90	70.65	-	81.56	73.76	-
ICMB 98004	101.35	111.49	-	84.12	76.56	-
ICMB 99022	83.81	77.62	-	87.34	79.75	-
Tift 23D_2B_1-P1-P5	84.17	67.66	79.24	91.89	85.59	72.07
P310-17-Bk	97.58	94.06	88.80	97.03	80.20	50.50
841B-P3	92.63	89.02	75.50	82.80	75.27	53.49
ICMB 95333	81.02	62.22	91.38	101.57	107.87	115.22
ICML 22	92.41	83.00	95.51	103.50	107.50	76.00
Mean	105.49	96.97	91.19	110.38	97.35	70.51
Std. Deviation	29.64	46.66	77.96	57.46	40.67	46.68
Std. Error	5.71	9.95	24.65	11.06	8.67	14.76

* Values not considered for salt tolerance comparisons as germination levels were negligible at that salt concentration.

Figure 4.8. Plots of responses between NaCl concentrations and root length (cm) of pearl millet seedlings from three groups of representative accessions



Note: (A) Sensitive (WSIL-P8, ICMB 90111), (B) moderately tolerant (863B-P2, 43B) and (C) highly tolerant (Tift23D_2B_1-P1-P5, 841B-P3)]

(Table 4.7). There were 15 accessions in this category. The relative survival % with respect to the controls in this group varied from 36.1% in ICMB 89111 to 94.9% in 843B. There were no leaf damage symptoms on any of the seedlings. These lines were categorized as 'moderately tolerant' to salinity stress.

3. The third category was comprised of pearl millet lines where seeds germinated without any symptoms of salt injury or morphological defects in the salt media up to 150mM NaCl concentration. The shoot dry matter production of these lines in 75mM NaCl ranged from 97.6% in P310-17-Bk and 81% in ICMB 95333, relative to their respective controls (Table 4.7). There were five accessions in this category among which, Tift $23D_2B_1$-P1-P5 was found as the most promising salt-tolerant line as it germinated up to 85.3% of its non-saline control value in 150mM NaCl. These were the 'highly tolerant' lines identified for salt stress tolerance during germination and early seedling growth in pearl millet.

The categorization of pearl millet inbred lines as sensitive, moderately tolerant and highly tolerant was based on their germination levels (Fig. 4.1) and early seedling growth (Plate 1) without any visible morphological defects up to NaCl levels of 75 mM, 100 mM and 150 mM, respectively. Hence, seven of the pearl millet inbred lines were categorized as sensitive (ICMB 90111, PRLT 2/89-33, P1449-2-P1, Tift $238D_1$-P152, 81B-P6, WSIL-P8 and ICMP 85410-P7), eleven as moderately tolerant, and five as highly tolerant (Tift $23D_2B_1$-P1-P5, ICMB 841-P3, P310-17-Bk, ICML 22, and ICMB 95333) (Table 4.8). The high level of dormancy observed in the seedlot of IP 18293-P158 prevented evaluation of its salinity tolerance in this study.

4.1.2 Biochemical parameters

4.1.2.1 Proline estimation

It was not previously known whether pearl millet accumulates osmolytes such as proline under salt stress or not. Free proline levels were noticed to increase with an increase in salinity levels (Figure 4.9) in all pearl millet lines included in this study. Proline accumulated according to the categories made above (based

Plate 1. Ten-day-old seedlings of pearl millet lines of (A) WSIL-P8, (B) ICMB 90111, (C) Tift 383 and (D) ICMB 99022 grown in 0mM, 75mM, 100mM and 150mM NaCl media

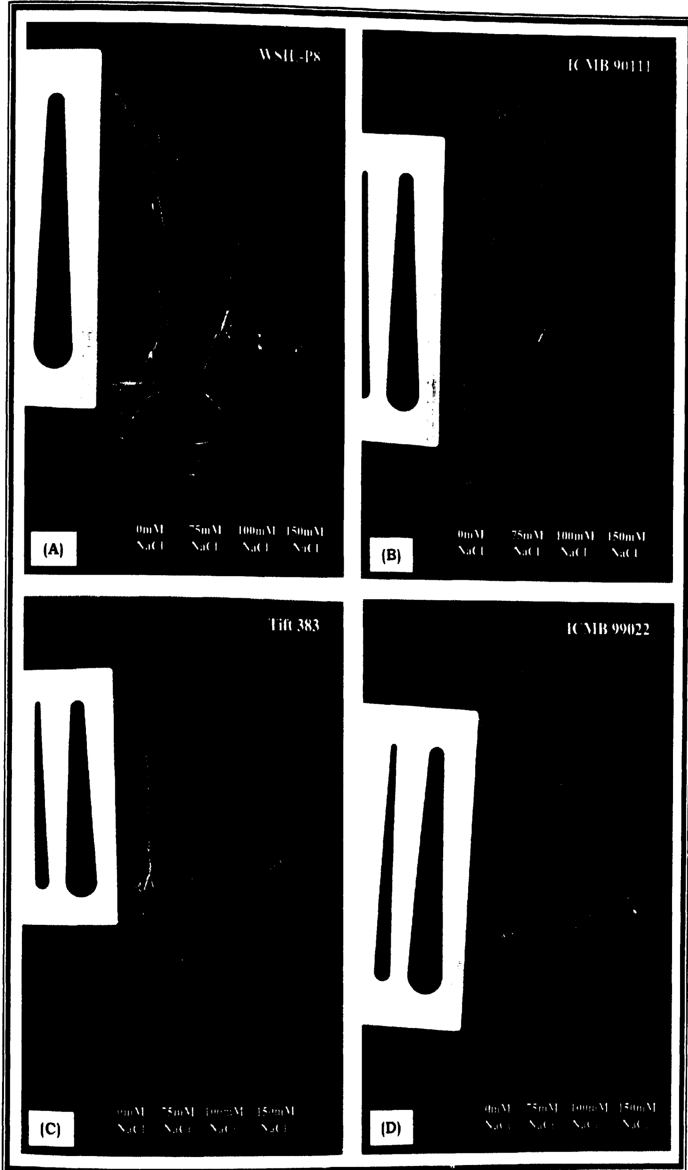
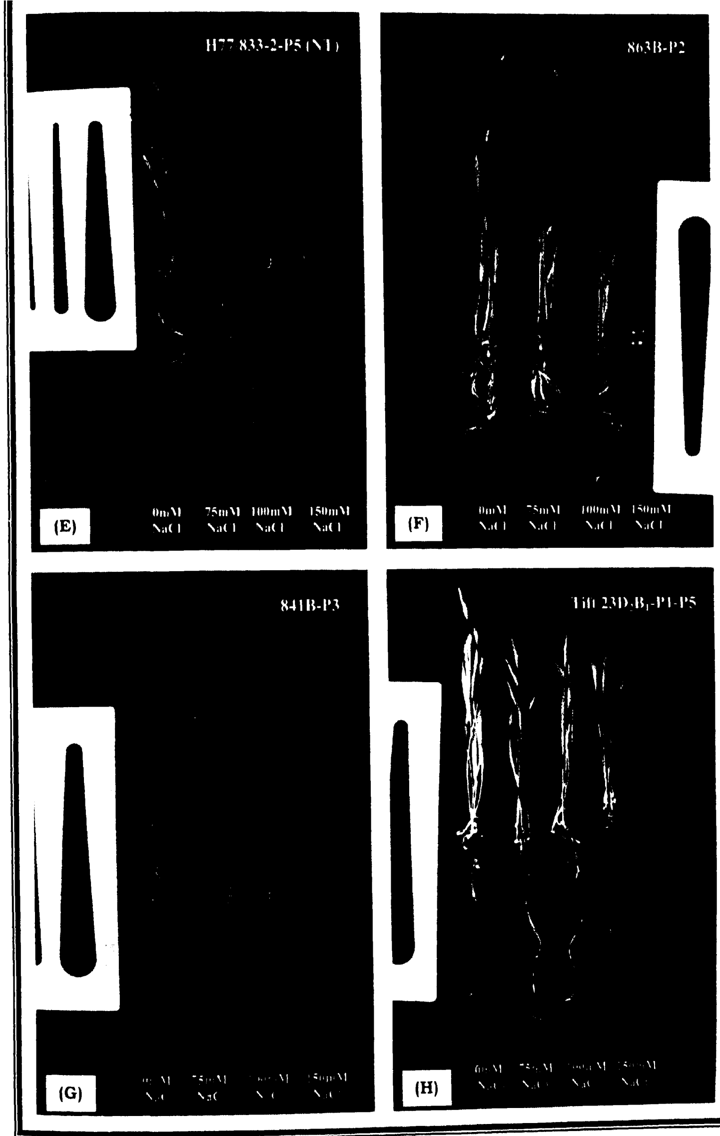


Plate 1. (contd.) Ten-day-old seedlings of pearl millet lines of (E) H77/833-2-P5 (NT), (F) 863B-P2, (G) 841B-P3 and (H) Tift $23D_2B_1$-P1-P5 grown in 0mM, 75mM, 100mM and 150mM NaCl media



[Note: Root growth is more affected than shoot length].

Table 4.8 Pearl millet lines categorized for response to salinity stress.

Entry number	Entry name	Response to salinity
1	**ICMP 85410-P7**	Sensitive
2	**WSIL-P8**	Sensitive
3	**81B-P6**	Sensitive
4	**PRLT 2/89-33**	Sensitive
5	**P1449-2-P1**	Sensitive
6	**ICMB 90111**	Sensitive
7	**Tift 238D_1-P158**	Sensitive
8	**IP 18293-P152**	*
9	**ICMP 451-P6**	Moderately tolerant
10	**ICMP 451-P8**	Moderately tolerant
11	**H 77/833-2**	Moderately tolerant
12	**H 77/833-2-P5(NT)**	Moderately tolerant
13	**W 504-1-P1**	Moderately tolerant
14	**PT 732B-P2**	Moderately tolerant
15	**863B-P2**	Moderately tolerant
16	**LGD 1-B-10**	Moderately tolerant
17	**Tift 186**	Moderately tolerant
18	**Tift 383**	Moderately tolerant
19	**ICMB 89111**	Moderately tolerant
20	**ICMB 92666**	Moderately tolerant
21	**843B**	Moderately tolerant
22	**ICMB 98004**	Moderately tolerant
23	**ICMB 99022**	Moderately tolerant
24	**Tift 23D_2B_1-P1-P5**	Highly tolerant
25	**P310-17-Bk**	Highly tolerant
26	**841B-P3**	Highly tolerant
27	**ICMB 95333**	Highly tolerant
28	**ICML 22**	Highly tolerant

* Dormancy or poor germination of the available seed stocks prevented effective assessment of salinity sensitivity/tolerance of this line.

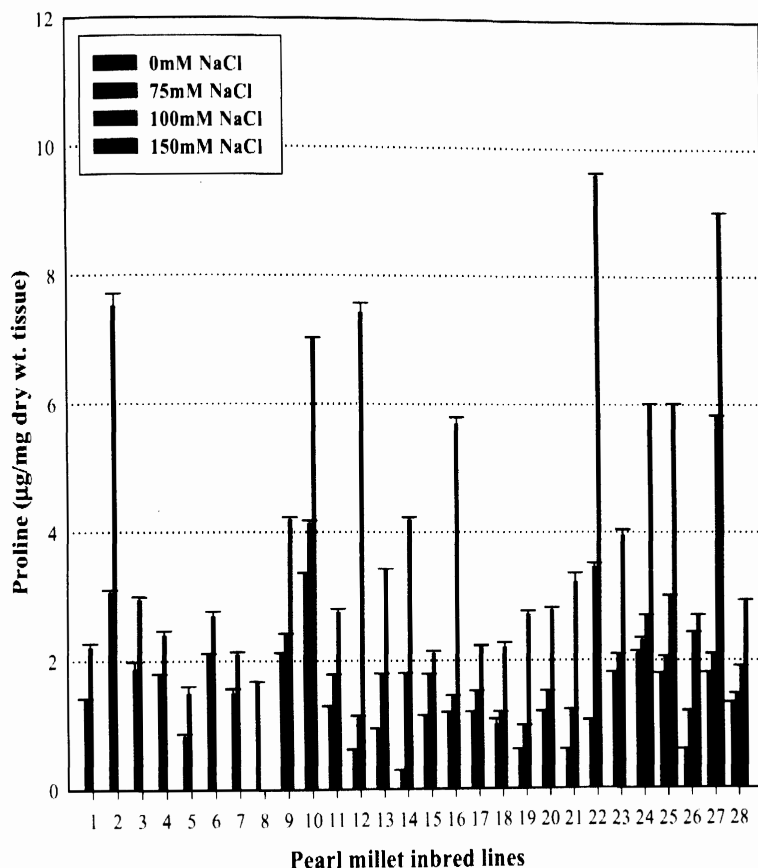
on growth of seedlings during salt stress) and also based on the increasing intensity of salt stress. Significantly greater increase in proline accumulation in the tolerant lines was observed in response to increasing salinity levels than in the sensitive lines. Among the sensitive lines (except for WSIL-P8 which exhibited a high proline accumulation at 75mM NaCl), there was only a slight increase in proline content at 75mM NaCl compared to the 0mM NaCl control. Moderately tolerant lines showed a varying response in proline accumulation. While genotype 863B-P2 accumulated the lowest level of proline observed at 100mM NaCl, ICMB 98004 accumulated the highest observed content of proline at this salinity level. However, in this category all the lines showed increases in proline accumulation with increasing salt stress. Among the highly tolerant lines, ICMB 95333 was seen to accumulate proline in very high amounts at 150mM, followed by Tift $23D_2B_1$-P1-P5 and P310-17-Bk. In general, there was a higher increase in accumulation of proline with increases in salinity in this category as compared to lines in the other two categories.

To ascertain the proline accumulation at high NaCl levels, free proline levels were estimated for a subset of pearl millet seedlings grown at different NaCl concentrations (0mM, 75mM, 100mM and 150mM). The representative salt-sensitive lines viz. WSIL-P8 and ICMB 90111 were seen to accumulate moderate levels of proline (5.1 and 3.15μg/mg dry weight at 100mM NaCl) and then it declined slightly (Figure 4.10A). The moderately salt-tolerant lines; 863B-P2 and 843B (3.56 and 4.2μg/mg dry weight) and highly salt-tolerant lines Tift $23D_2B_1$-P1-P5 and 841B-P3 showed an increase in proline accumulation with an increase in NaCl concentrations (6 and 3.69μg/mg dry weight) till 150mM NaCl levels (Figure 4.10 B and C).

4.1.2.2 Estimation of Na^+ and K^+ ions under salt stress

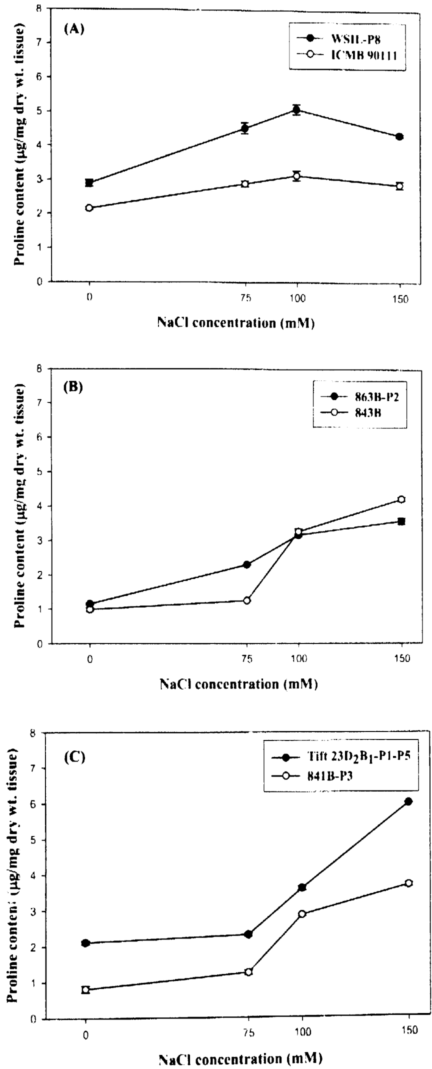
The mechanism of sequestering ions under salt stress into the vacuoles was not known yet for pearl millet. It was also not known if K^+ ion is accumulated in pearl millet to counter Na^+ toxicity and also for ion homeostasis. Estimation of ion concentrations in salt treated seedlings and controls would give clues about this. Among the 27 germinating entries tested, most of them showed an

Figure 4.9. Proline content (µg/mg dry wt. tissue) under non-saline conditions and three levels of salt stress in 28 inbred lines of pearl millet



[Inbred lines: (1) ICMP 85410-P7, (2) WSIL-P8, (3) 81B-P6, (4) PRLT 2/89-33, (5) P1449-2-P1, (6) ICMB 90111, (7) Tift 238D$_1$-P158, (8) IP 18293-P152, (9) ICMP 451-P6, (10) ICMP 451-P8, (11) H 77/833-2, (12) H 77/833-2-P5 (NT), (13) W 504-1-P1, (14) PT 732B-P2, (15) 863B-P2, (16) LGD 1-B-10, (17) Tift 186, (18) Tift 383, (19) ICMB 89111, (20) ICMB 92666, (21) 843B, (22) ICMB 98004, (23) ICMB 99022, (24) Tift 23D$_2$B$_1$-P1-P5, (25) P310-17-Bk, (26) 841B-P3, (27) ICMB 95333, (28) ICML 22]

Figure 4.10. Plots of responses between NaCl concentrations and proline content (µg/mg dry wt. tissue) of pearl millet seedlings from three groups of representative accessions



Note: (A) Sensitive (WSIL-P8, ICMB 90111), (B) moderately tolerant (863B-'2, 843B) and (C) highly tolerant (Tift $23D_2B_1$-P1-P5, 841B-P3)]

increase in Na^+ content under salt stress. The entries that failed to germinate in each salinity level were not included for ion estimation at that particular salinity level (Figure 4.11). At 75mM NaCl, accumulation of Na^+ in sensitive lines was in the range of that of the moderately tolerant and highly tolerant lines [Figure 4.11(A)]. Most of the sensitive lines showed higher accumulation of K^+ at 75mM NaCl than did the moderately and highly tolerant lines [Figure 4.11(B)]. Also, K^+/Na^+ ratios [Figure 4.11(C)] were higher than the other two categories at 0mM and 75mM NaCl in these seven salinity sensitive lines. In two of the lines viz. ICMP 85410-P7 and 81B-P6 this ratio decreased indicating relatively less accumulation of K^+ than Na^+ at the higher salinity level. In rest of these lines, increasing salinity levels resulted in relatively increased accumulation of K^+.

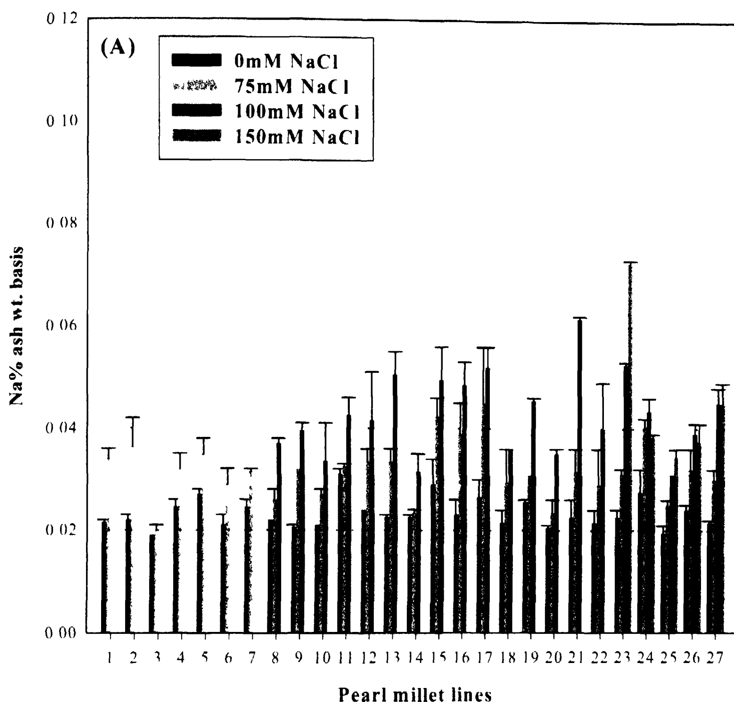
Among the moderately tolerant inbred lines, Na and K^+ levels varied considerably across the different salinity levels. Most of the lines in this category showed a positive relationship between salt concentration and uptake of Na^+ and K^+. Of these lines ICMB 98004 had conspicuously reduced K^+/Na^+ ratios with increased salinity levels. Other lines that exhibited similar trends were ICMP 451-P6, PT 732B-P2 and ICMB 92666; while the rest of the lines accumulated more K^+ than Na^+ with increasing salt stress.

Of the five tolerant lines, Tift $23D_2B_1$-P1-P5 accumulated the highest levels of Na^+ as well as K^+. The K^+/Na^+ ratio was quite low in this line; however, it increased gradually with increases in salinity levels of the media. In the tolerant lines P310-17-Bk and 841B-P3, K^+/Na^+ ratio increased substantially, and significantly, at the higher media salinity levels. Thus, the 27 inbred lines in this study differed markedly in their ion uptake behavior in response to increased salinity especially in terms of the ratio of K^+ over Na^+.

4.1.2.3 Effect of short-term salt stress on accumulation of proline

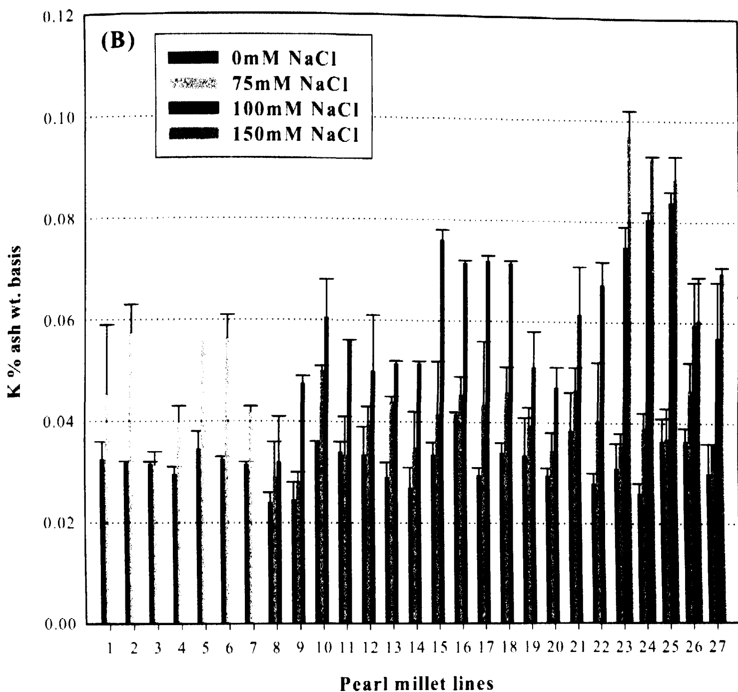
Ten-day-old seedlings of pearl millet lines representing the three categories, sensitive (WSIL-P8 and ICMB 90111), moderately tolerant (863B-P2 and 843B) and highly tolerant (Tift $23D_2B_1$-P1-P5 and 841B-P3) were subjected to 150mM NaCl for 0h, 24h, 48h, 72h and 96h. Accumulation of proline monitored for

Figure 4.11. (A) Na^+ content (% ash wt. basis) in salt treated seedlings of 27 pearl millet inbred lines as compared to their controls



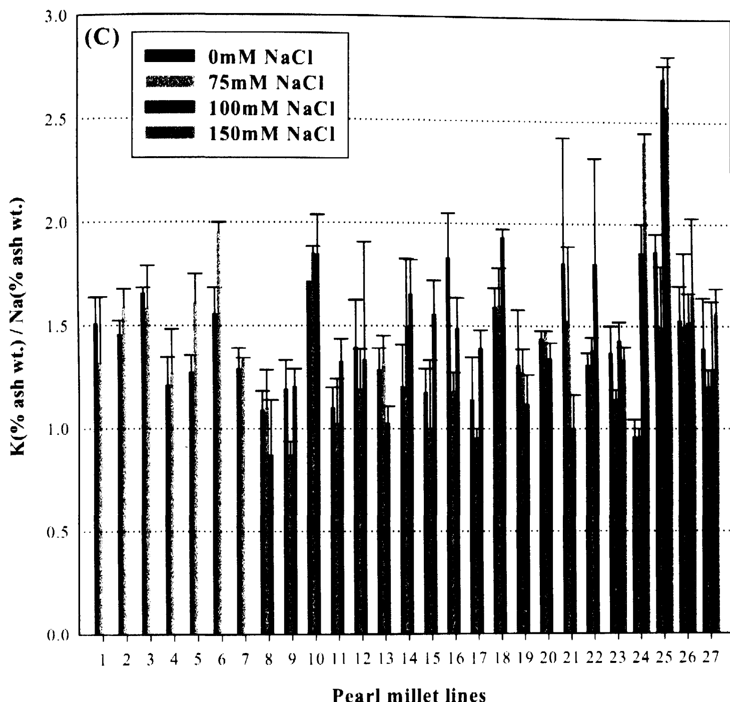
[(1) ICMP 85410-P7, (2) WSIL-P8, (3) 81B-P6, (4) PRLT 2/89-33, (5) P1449-2-P1, (6) ICMB 90111, (7) Tift $238D_1$-P158, (8) ICMP 451-P6, (9) ICMP 451-P8, (10) H 77/833-2, (11) H 77/833-2-P5 (NT), (12) W 504-1-P1, (13) PT 732B-P2, (14) 863B-P2, (15) LGD 1-B-10, (16) Tift 186, (17) Tift 383, (18) ICMB 89111, (19) ICMB 92666, (20) 843B, (21) ICMB 98004, (22) ICMB 99022, (23) Tift $23D_2B_1$-P1-P5, (24) P310-17-Bk, (25) 841B-P3, (26) ICMB 95333, (27) ICML 22]

igure 4.11. (B) K^+ content (% ash wt. basis) in salt treated seedlings of 27 earl millet inbred lines as compared to their controls



[(1) ICMP 85410-P7, (2) WSIL-P8, (3) 81B-P6, (4) PRLT 2/89-33, (5) P1449-2-P1, (6) ICMB 90111, (7) Tift $238D_1$-P158, (8) ICMP 451-P6, (9) ICMP 451-P8, (10) H 77/833-2, (11) H 77/833-2-P5 (NT), (12) W 504-1-P1, (13) PT 732B-P2, (14) 863B-P2, (15) LGD 1-B-10, (16) Tift 186, (17) Tift 383, (18) ICMB 89111, (19) ICMB 92666, (20) 843B, (21) ICMB 98004, (22) ICMB 99022, (23) Tift $23D_2B_1$-P1-P5, (24) P310-17-Bk, (25) 841B-P3, (26) ICMB 95333, (27) ICML 22]

igure 4.11. (C) K^+/Na^+ ratios in salt treated seedlings of 27 pearl millet ɑbred lines as compared to their controls



[(1) ICMP 85410-P7, (2) WSIL-P8, (3) 81B-P6, (4) PRLT 2/89-33, (5) P1449-2-P1, (6) ICMB 90111, (7) Tift $238D_1$-P158, (8) ICMP 451-P6, (9) ICMP 451-P8, (10) H 77/833-2, (11) H 77/833-2-P5(OT), (12) W 504-1-P1, (13) PT 732B-P2, (14) 863B-P2, (15) LGD 1-B-10, (16) Tift 186, (17) Tift 383, (18) ICMB 89111, (19) ICMB 92666, (20) 843B, (21) ICMB 98004, (22) ICMB 99022, (23) Tift $23D_2B_1$-P1-P5, (24) P310-17-Bk, (25) 841B-P3, (26) ICMB 95333, (27) ICML 22]

these short-time treatments indicated that the constitutive levels were higher in the salt susceptible lines compared to the tolerant ones. The increase in proline levels was substantially lower compared to the corresponding controls in the salt-sensitive lines (3.52 μg/mg compared to 2.75μg/mg dry weight at 96h in the accession WSIL-P8 and 2.75μg/mg compared to 2.25μg/mg dry weight in the line ICMB 90111 at 96h) than in the moderately tolerant lines (Table 4.9 A and B). The fold-wise increase at 96h in the proline content between control and treatments were about 3 in both the moderately lines 863B-P2 and 843B (Table 4.9B). In the highly tolerant lines (especially in 841B-P3), the proline levels marginally increased at 24h and 48h compared to the control values, and thereafter increased considerably (2 to 6-folds) at 72h and 96h to the values comparatively higher than the sensitive and moderately tolerant lines (Table 4.9C). These results are indicative of the fact that proline accumulation may be a possible mechanism conferring salinity tolerance in pearl millet.

4.1.2.4 Effect of short-term salt stress on accumulation of ions

Accumulation of ions (Na^+, K^+, Ca^{2+}, Cl^-) were measured in 10-day-old seedlings of five representative accessions of pearl millet, which were subjected to short-term salt stress at 150mM NaCl for 0h, 24h, 48h, 72h, 96h, 120h and 144h. Increasing magnitude of salt exposure led to an increase in the content of Na^+, K^+ and Cl^- ions in the pearl millet lines (Table 4.10 A-E). The accumulation of Na^+ was slightly more in the susceptible lines compared to the moderately tolerant and tolerant lines. Contrary to it, the accumulation of K^+ in the susceptible varieties were nearly 3-folds (Table 4.10A,B,C), while it was 5 to 7-folds in the highly tolerant accessions (Table 4.10D,E). Lines WSIL-P8 and ICMB 90111 displayed marginal increase in the accumulation of Ca^{2+}, but was much higher in the tolerant lines especially at longer salt exposure (120h and 144h). Line 841B-P3 showed the highest accumulation of Ca^{2+} with increasing exposure to salinity). However, the increase in Cl^- with exposure to salinity was higher in the sensitive lines WSIL-P8 and ICMB 90111, than in moderately tolerant 863B-P2 and highly tolerant, Tift $23D_2B_1$-P1-P5 and 841B-P3.

Table 4.9. Proline content (µg/mg dry wt. tissue) under short-term salt stress at 150mM NaCl from three groups of representative accessions of pearl millet seedlings; (A) sensitive (WSIL-P8, ICMB 90111), (B) moderately tolerant (863B-P2, 843B) and (C) highly tolerant (Tift $23D_2B_1$-P1-P5, 841B-P3)

(A)

Time in Hours	Proline content (µg/mg dry wt. tissue) *			
	WSIL-P8		ICMB 90111	
	Control	150mM NaCl	Control	150mM NaCl
0	2.70 (±0.51)	2.85 (±0.24)	2.21 (±0.18)	2.23 (±0.28)
24	2.72 (±0.22)	2.93 (±0.61)	2.15 (±0.33)	2.35 (±0.19)
48	2.72 (±0.12)	3.51 (±0.61)	2.19 (±0.41)	2.75 (±0.29)
72	2.69 (±0.93)	3.60 (±0.25)	2.23 (±0.25)	2.59 (±0.56)
96	2.75 (±0.53)	3.52 (±0.19)	2.25 (±0.21)	2.75 (±0.36)

Time in Hours	Proline content (µg/mg dry wt. tissue) *			
	863B-P2		843B	
	Control	150mM NaCl	Control	150mM NaCl
0	1.19 (±0.12)	1.21(±0.15)	1.02 (±0.15)	1.03 (±0.61)
24	1.25 (±0.35)	1.56 (±0.43)	0.99 (±0.12)	1.35 (±0.41)
48	1.16 (±0.36)	2.89 (±0.22)	1.15 (±0.28)	2.99 (±0.50)
72	1.21 (±0.21)	3.58 (±0.16)	1.12 (±0.15)	4.02 (±0.52)
96	1.25 (±0.90)	3.65 (±0.71)	1.15 (±0.21)	3.98 (±0.75)

Time in Hours	Proline content (µg/mg dry wt. tissue) *			
	Tift $23D_2B_1$-P1-P5		841B-P3	
	Control	150mM NaCl	Control	150mM NaCl
0	2.10 (±0.55)	2.15 (±0.18)	0.55 (±0.58)	0.61 (±0.91)
24	2.12 (±0.25)	2.19 (±0.36)	0.52 (±0.65)	0.90 (±0.57)
48	2.18 (±0.13)	2.88 (±0.92)	0.63 (±0.20)	1.95 (±0.55)
72	2.15 (±0.68)	3.65 (±0.81)	0.65 (±0.12)	2.79 (±0.15)
96	2.18 (±0.20)	5.75 (±0.66)	0.59 (±0.15)	4.05 (±0.10)

* Data represent mean of three replicates

* Values in the parenthesis indicate the standard errors

* P value < 0.05

Table 4.10. Ion content (mg/g dry wt.) from three groups of representative accessions of pearl millet seedlings under short-term salt stress at 150mM NaCl; (A) WSIL-P8, (B) ICMB 90111, (C) 863B-P2, (D) Tift $23D_2B_1$-P1-P5 and (E) 841B-P3

(A)

Ion content (mg/g dry wt.) *								
WSIL-P8								
Time in Hours	Na^+		K^+		Ca^{2+}		Cl^-	
	0mM NaCl	150mM NaCl	0mM NaCl	150mM NaCl	0mM NaCl	150mM NaCl	0mM NaCl	150mM NaCl
0	0.025 (±0.7)	0.025 (±0.9)	0.028 (±0.2)	0.029 (±0.5)	0.025 (±0.4)	0.024 (±0.60)	0.043 (±0.1)	0.045 (±0.80)
24	0.026 (±0.5)	0.036 (±0.9)	0.029 (±0.3)	0.037 (±0.4)	0.024 (±0.1)	0.019 (±0.80)	0.043 (±0.7)	0.067 (±0.35)
48	0.025 (±0.3)	0.044 (±1.1)	0.027 (±0.5)	0.042 (±0.7)	0.025 (±0.1)	0.019 (±0.60)	0.045 (±0.3)	0.079 (±1.2)
72	0.027 (±0.9)	0.055 (±0.6)	0.030 (±0.3)	0.056 (±0.8)	0.026 (±0.3)	0.018 (±0.60)	0.044 (±0.9)	0.118 (±0.75)
96	0.028 (±0.8)	0.077 (±0.8)	0.028 (±0.5)	0.072 (±0.3)	0.027 (±0.2)	0.019 (±0.45)	0.046 (±0.5)	0.146 (±1.00)
120	0.027 (±0.9)	0.105 (±0.3)	0.031 (±0.2)	0.071 (±0.8)	0.027 (±0.9)	0.020 (±0.50)	0.047 (±0.6)	0.136 (±0.25)
144	0.028 (±1.0)	0.115 (±0.2)	0.029 (±0.9)	0.089 (±0.5)	0.028 (±0.9)	0.025 (±0.25)	0.049 (±0.2)	0.174 (±0.45)

(B)

Ion content (mg/g dry wt.) *								
ICMB 90111								
Time in Hours	Na^+		K^+		Ca^{2+}		Cl^-	
	0mM NaCl	150mM NaCl	0mM NaCl	150mM NaCl	0mM NaCl	150mM NaCl	0mM NaCl	150mM NaCl
0	0.019 (±0.0)	0.021 (±0.3)	0.018 (±0.3)	0.022 (±0.9)	0.021 (±0.5)	0.020 (±0.6)	0.098 (±0.1)	0.100 (±0.5)
24	0.021 (±0.3)	0.029 (±1.1)	0.020 (±0.5)	0.021 (±0.3)	0.019 (±0.6)	0.018 (±0.3)	0.095 (±0.1)	0.121 (±0.0)
48	0.020 (±0.5)	0.048 (±0.5)	0.022 (±0.9)	0.023 (±0.2)	0.020 (±0.7)	0.020 (±0.4)	0.105 (±0.2)	0.156 (±0.1)
72	0.021 (±0.5)	0.062 (±0.4)	0.023 (±0.4)	0.041 (±0.5)	0.022 (±0.3)	0.017 (±0.9)	0.106 (±0.6)	0.126 (±0.2)
96	0.022 (±0.6)	0.072 (±0.3)	0.025 (±0.5)	0.054 (±0.5)	0.019 (±0.9)	0.016 (±0.2)	0.106 (±0.3)	0.128 (±0.3)
120	0.024 (±0.2)	0.078 (±0.1)	0.026 (±0.6)	0.059 (±0.9)	0.018 (±0.9)	0.019 (±0.1)	0.108 (±0.8)	0.134 (±0.9)
144	0.025 (±0.5)	0.082 (±0.4)	0.024 (±0.2)	0.069 (±0.8)	0.020 (±0.7)	0.021 (±0.5)	0.110 (±0.5)	0.142 (±0.2)

Table 4.10 contd.

(C)

Ion content (mg/g dry wt.) *								
863B-P2								
Time in Hours	Na^+		K^+		Ca^{2+}		Cl^-	
	0mM NaCl	150mM NaCl	0mM NaCl	150mM NaCl	0mM NaCl	150mM NaCl	0mM NaCl	150mM NaCl
0	0.029 (±0.2)	0.031 (±1.2)	0.021 (±1.0)	0.023 (±1.0)	0.020 (±0.5)	0.022 (±0.1)	0.085 (±0.4)	0.084 (±0.4)
24	0.029 (±0.2)	0.050 (±0.6)	0.023 (±0.5)	0.039 (±0.4)	0.021 (±1.2)	0.019 (±0.2)	0.082 (±0.6)	0.083 (±0.8)
48	0.031 (±0.6)	0.055 (±0.3)	0.022 (±0.3)	0.055 (±0.7)	0.019 (±0.9)	0.019 (±0.4)	0.085 (±0.6)	0.103 (±0.2)
72	0.030 (±0.7)	0.059 (±0.8)	0.023 (±0.3)	0.053 (±0.9)	0.017 (±0.7)	0.020 (±0.1)	0.089 (±0.8)	0.096 (±0.1)
96	0.031 (±0.1)	0.064 (±0.5)	0.025 (±0.2)	0.079 (±0.2)	0.018 (±0.8)	0.020 (±0.4)	0.088 (±0.3)	0.088 (±0.9)
120	0.032 (±0.9)	0.077 (±0.2)	0.025 (±0.5)	0.120 (±0.5)	0.020 (±0.8)	0.037 (±0.0)	0.090 (±0.4)	0.091 (±0.2)
144	0.034 (±0.2)	0.086 (±0.3)	0.026 (±0.2)	0.159 (±0.3)	0.022 (±0.2)	0.032 (±0.2)	0.091 (±0.3)	0.106 (±0.4)

(D)

Ion content (mg/g dry wt.) *								
Tift $23D_2B_1$-P1-P5								
Time in Hours	Na^+		K^+		Ca^{2+}		Cl^-	
	0mM NaCl	150mM NaCl	0mM NaCl	150mM NaCl	0mM NaCl	150mM NaCl	0mM NaCl	150mM NaCl
0	0.025 (±0.6)	0.027 (±0.2)	0.022 (±0.7)	0.025 (±0.3)	0.017 (±0.8)	0.017 (±0.9)	0.042 (±1.0)	0.041 (±0.5)
24	0.025 (±0.5)	0.056 (±0.4)	0.025 (±0.1)	0.027 (±0.4)	0.015 (±0.3)	0.019 (±0.2)	0.042 (±0.5)	0.044 (±0.2)
48	0.027 (±0.8)	0.090 (±0.5)	0.025 (±0.9)	0.037 (±0.9)	0.015 (±0.4)	0.023 (±0.5)	0.043 (±0.3)	0.049 (±0.7)
72	0.029 (±0.3)	0.093 (±0.4)	0.024 (±0.2)	0.062 (±0.2)	0.017 (±1.2)	0.022 (±0.3)	0.045 (±0.3)	0.118 (±0.2)
96	0.030 (±0.4)	0.093 (±0.7)	0.026 (±0.8)	0.091 (±0.1)	0.016 (±0.9)	0.026 (±0.3)	0.043 (±0.2)	0.139 (±0.3)
120	0.032 (±0.8)	0.102 (±0.2)	0.028 (±0.3)	0.117 (±0.7)	0.019 (±0.5)	0.043 (±1.2)	0.048 (±0.5)	0.147 (±0.1)
144	0.031 (±0.9)	0.129 (±0.6)	0.031 (±0.4)	0.153 (±0.6)	0.018 (±0.5)	0.063 (±0.9)	0.052 (±0.2)	0.154 (±0.5)

Table 4.10 contd.

(E)

Ion content (mg/g dry wt.) *								
841B-P3								
Time in Hours	**Na^+**		**K^+**		**Ca^{2+}**		**Cl^-**	
	0mM NaCl	**150mM NaCl**	**0mM NaCl**	**150mM NaCl**	**0mM NaCl**	**150mM NaCl**	**0mM NaCl**	**150mM NaCl**
0	0.021 (±0.5)	0.023 (±0.23)	0.029 (±0.5)	0.031 (±0.65)	0.018 (±1.1)	0.022 (±0.35)	0.045 (±0.7)	0.046 (±0.60)
24	0.020 (±0.3)	0.032 (±0.95)	0.029 (±1.2)	0.058 (±0.90)	0.020 (±0.9)	0.042 (±0.65)	0.048 (±0.1)	0.071 (±0.15)
48	0.023 (±0.3)	0.067 (±0.99)	0.032 (±0.9)	0.067 (±0.35)	0.020 (±1.2)	0.066 (±0.75)	0.052 (±0.9)	0.075 (±0.60)
72	0.025 (±0.4)	0.071 (±0.45)	0.033 (±0.7)	0.076 (±0.65)	0.023 (±0.7)	0.072 (±0.65)	0.051 (±0.2)	0.076 (±0.50)
96	0.026 (±0.9)	0.080 (±0.85)	0.035 (±0.8)	0.089 (±0.66)	0.026 (±0.8)	0.097 (±0.65)	0.056 (±0.8)	0.078 (±0.55)
120	0.028 (±0.2)	0.088 (±0.95)	0.039 (±0.8)	0.129 (±0.37)	0.032 (±0.6)	0.087 (±0.90)	0.059 (±0.3)	0.085 (±0.34)
144	0.031 (±0.8)	0.078 (±0.35)	0.042 (±0.2)	0.153 (±0.95)	0.037 (±0.5)	0.104 (±0.59)	0.064 (±0.4)	0.105 (±1.00)

*** Data represent mean of three replicates.**
*** Values in the parenthesis indicate the standard errors.**
*** P value < 0.05.**

4.2 Antioxidative enzyme activity during short-term salt stress

4.2.1 Antioxidative enzyme activities of three representative lines of pearl millet

The pearl millet lines ICMB 90111, 863B-P2 and 841B-P3; which were categorized as sensitive, moderately tolerant and highly tolerant to salinity stress, respectively, were exposed to 150mM NaCl salt stress for short intervals of time i.e., 0h, 24h, 48h, 72h, 96h, 120h and 144h. The modulation of antioxidant components diverged significantly among these three representative lines of pearl millet under salt stress conditions as shown in Figure 4.12 (A-F)].

4.2.1.1 Catalase (CAT)

Specific activity of catalase decreased in the sensitive line ICMB 90111 under short-term salt stress at 150mM NaCl, but the activity doubled by 72h. It declined thereafter till 144h (Figure 4.12A). On the other hand, slight increase in the activities was noticed in 863B-P2 till 96h, which dropped afterwards. In 841B-P3, the activity peaked 12-folds by 72h but declined thereafter [Figure 4.12(A).

4.2.1.2 Superoxide dismutase (SOD)

The native activity of superoxide dismutase appeared lower in the sensitive line when compared to moderately tolerant and tolerant lines [Figure 4.12B). A sudden spurt (about 3.5-folds) in SOD activity at 72h was observed in ICMB 90111 coinciding with a spurt in CAT activity. It gradually decreased with time of exposure to 150mM NaCl till 144h. At 144h, this line expressed lower SOD activity than the other two lines (Figure 4.12B). Though 863B-P2 exhibited the highest SOD activity at 0h and 24h, it actually declined sporadically or fluctuated till 144h. In case of 841B-P3, SOD activity increased till 48h, declined till 96h but it picked up again slightly. At 144h, this salinity tolerant line recorded the highest SOD activity compared to the other lines (Figure 4.12B).

4.2.1.3 Glutathione reductase (GR)

The native activity of glutathione reductase (GR) was lower in salinity-sensitive line ICMB 90111 than in the moderately tolerant and tolerant lines [Figure

4.12C). The activities of GR did not show any specific trends in these lines. The tolerant varieties however, recorded higher activities than the susceptible ones at 144h (Figure 4.12C).

4.2.1.5 Glutathione-S-transferase (GST)

Activity of GST declined initially (24h) but picked up slightly by 72h. The specific activity however decreased thereafter in ICMB 90111 (Figure 4.12D). GST activity decreased initially (48h) but only to increase slowly and steadily till 120h in 863B-P2. Thus the highest GST activity was noticed in moderately tolerant line 863B-P2, though the levels fluctuated. In the case of tolerant variety the activity enhanced by 48h but declined thereafter till 144h (Figure 4.12D).

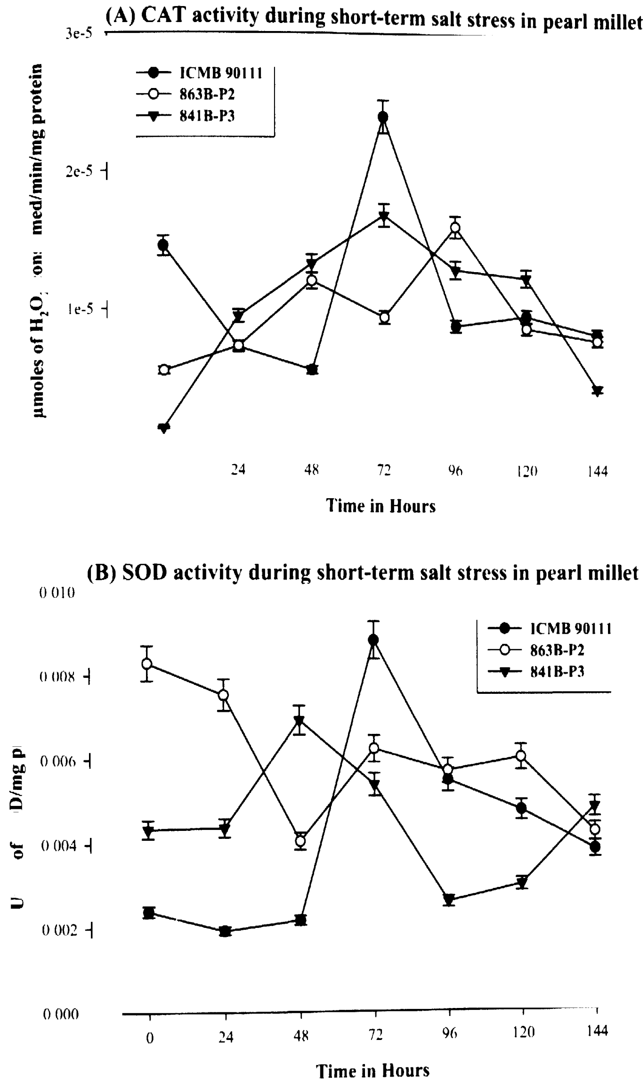
4.2.1.4 Reduced Glutathione (GSH) content

The content of reduced glutathione (GSH) is shown in Figure 4.12E. The content of GSH did not change much in the line ICMB 90111 till 48h though it declined later on. In contrast, an increase in GSH was observed with increased time of exposure to salinity stress in 863B-P2 till 48h. It declined by 72h but rose sharply by 96h. However, the content decreased towards the termination of the treatment (Figure 4.12E). The content of GSH did not change in the highly tolerant line 841B-P3 (Figure 4.12E).

4.2.1.5 Lipid peroxidation

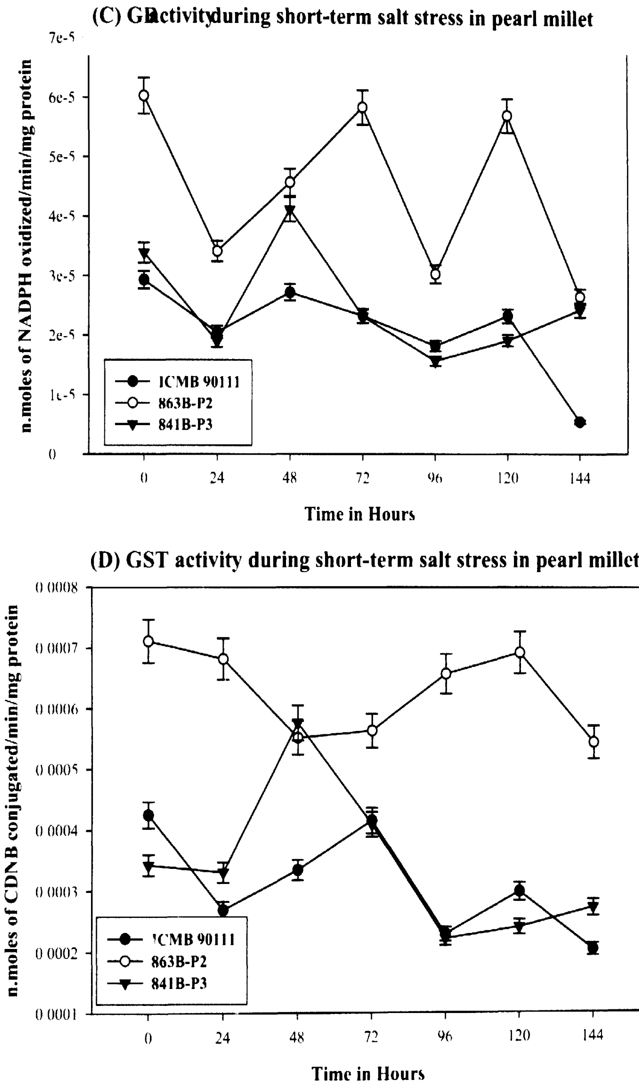
MDA levels increased slightly in the sensitive line ICMB 90111 by 96h and then declined later on. The content fluctuated and did not show any specific trend in the line 863B-P2. The content of MDA peaked by 144h in this line. The content of MDA increased by 4-5-folds in the tolerant line 841B-P3 till 96h which started declining later. It recorded the lowest content at 144h among the three lines (Figure 4.12F).

igure 4.12. Changes in antioxidative enzyme activity during short-term alt stress at 150mM NaCl in three representative lines of pearl millet. A) Catalase, (B) Superoxide dismutase



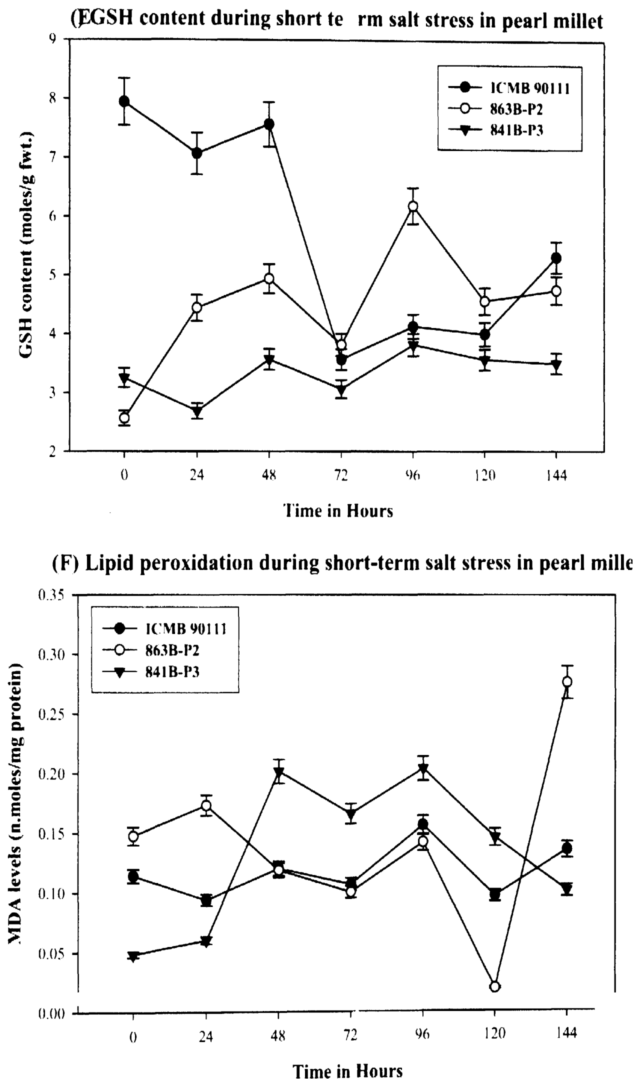
[Note: ICMB 90111 (sensitive), 863B-P2 (moderately tolerant) and 841B-P3 (highly tolerant)]

Figure 4.12. Changes in antioxidative enzyme activity during short-term salt stress at 150mM NaCl in three representative lines of pearl millet (C) Glutathione reductase, (D) Glutathione-S-transferase



[Note: ICMB 90111 (sensitive), 863B-P2 (moderately tolerant) and 841B-P3 (highly tolerant)]

Figure 4.12. Changes in (E) Reduced Glutathione content and (F) Lipid peroxidation during short-term salt stress at 150mM NaCl in three representative lines of pearl millet



[Note: ICMB 90111 (sensitive), 863B-P2 (moderately tolerant) and 841B-P3 (highly tolerant)]

4.2.2 Antioxidative enzyme activities of pearl millet lines under continuous salt stress for 7 days at different salinity levels

Pearl millet lines ICMB 90111, 863B-P2 and 841B-P3 that are sensitive, moderately tolerant and highly tolerant to salinity stress respectively, were exposed to 0mM, 75mM, 100mM and 150mM NaCl for 7 days. The antioxidant enzyme activities diverged significantly among these three pearl millet lines with increased intensity of salt stress (Figure 4.13A-F).

4.2.2.1 Catalase (CAT)

The activity of CAT in the control seedlings (grown without salt stress), was high in ICMB 90111 compared to the other two lines. Catalase activity did not change much at three different levels of salinity in the line ICMB 90111 though it showed enhanced activity compared to the control. The activity increased at 75 and 100mM NaCl levels but declined at higher NaCl stress. In the line 841B-P3, the activity increased at all the three levels of stress compared to the control (Figure 4.13A).

4.2.2.2 Superoxide dismutase (SOD)

Compared to the control, activity of SOD increased slightly at 75mM NaCl, but declined drastically with increasing concentrations of salt i.e., 100mM and 150mM NaCl in ICMB 90111 [Figure 4.13B]. The activity in moderately tolerant line declined at 75mM and 100mM NaCl but increased slightly at 150mM salt stress. Increased activity was noticed at 75mM NaCl in the tolerant line but it declined at higher concentrations (Figure 4.13B).

4.2.2.3 Glutathione reductase (GR)

Specific activity of glutathione reductase increased only at 75mM NaCl stress but declined at 100mM salt stress. But the activity doubled at 150mM salt stress in the line ICMB 90111. The increase was steady and doubled at 150mM NaCl stress in the moderately tolerant line. In the tolerant line 841B-P3, GR activity decreased considerably especially at higher salt stress conditions (Figure 4.13C).

4.2.2.4 Glutathione-S-transferase (GST)

The native activity of GST was higher in the salt susceptible line compared to the tolerant ones. However, the activity declined in the seedlings of all the three lines upon exposure to different concentrations of salt and this decline was more pronounced at 150mM NaCl in the tolerant line compared to the other lines (Figure 4.13D).

4.2.2.5 Reduced glutathione (GSH) content

The decline in the content of glutathione was significant at 75mM NaCl stress in the accession ICMB 90111. The content was slightly higher at 100 and 150mM NaCl stress but was less compared to the control seedlings. The glutathione content increased in presence of salt stress especially at 100mM NaCl stress in the moderately tolerant line. The content was the lowest at 75mM NaCl stress (declined by 3-folds) compared to 100mM (4.604 moles/g fwt.) and 150mM NaCl stress (5.208 moles/g fwt.) in the highly tolerant line 841B-P3 (Figure 4.13E).

4.2.2.6 Lipid peroxidation

Lipid peroxidation increased with an increase in the concentration of salt in the salt susceptible line ICMB 90111 [Figure 4.13F). In contrast, malondialdehyde (MDA) levels decreased considerably (2-6-folds) in 863B-P2. In the line 841B-P3, the content of MDA decreased with an increase in salinity (Figure 4.13F).

4.2.3 Antioxidative enzyme activities of parental lines of pearl millet with extreme responses to salt stress

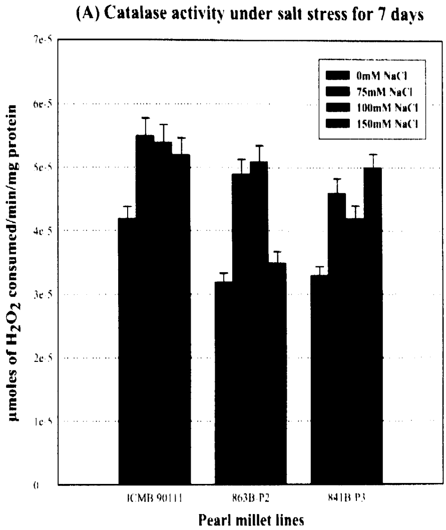
4.2.3.1 Catalase (CAT)

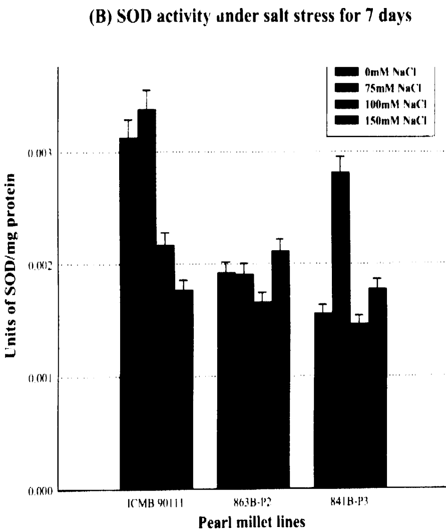
The specific activity of catalase was higher in the seedlings of the line Tift $23D_2B_1$-P1-P5 when compared to WSIL-P8. In general, the activity declined under salt stress and showed almost a similar trend (Figure 4.14A).

4.2.3.2 Superoxide dismutase (SOD)

Like catalase, the native activity of SOD was higher in the line Tift$23D_2B_1$-P1-P5 when compared to the tolerant line. The activity of SOD declined till 72h under salt stress but exhibited activity like that of control seedlings in the line

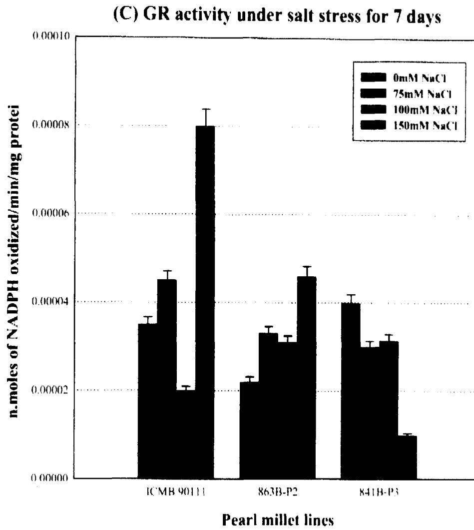
Figure 4.13 Changes in antioxidative enzyme activity during continuous salt stress for 7 days at different concentrations of NaCl in three representative lines of pearl millet [(A) Catalase, (B) Superoxide dismutase]

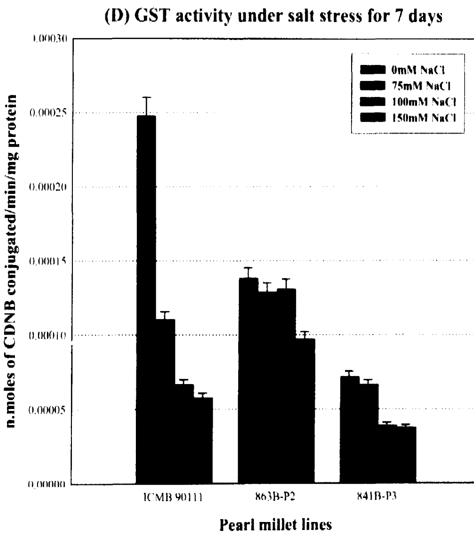




[Note: ICMB 90111 (sensitive), 863B-P2 (moderately tolerant) and 841B-P3 (highly tolerant)]

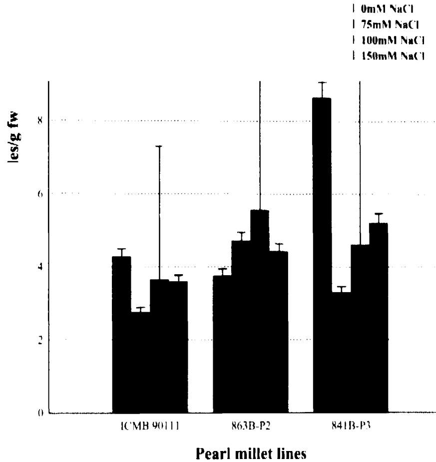
Figure 4.13 Changes in antioxidative enzyme activity during continuous salt stress for 7 days at different concentrations of NaCl in three representative lines of pearl millet [(C) Glutathione reductase, (D) Glutathione-S-transferase]

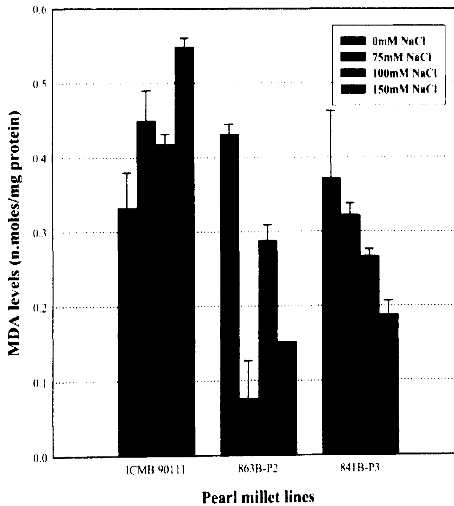




[Note: ICMB 90111 (sensitive), 863B-P2 (moderately tolerant) and 841B-P3 (highly tolerant)]

Fig. 4.13 Changes in (E) Reduced Glutathione content and (F) Lipid peroxidation during continuous salt stress for 7 days at different concentrations of NaCl in three representative lines of pearl millet





[Note: ICMB 90111 (sensitive), 863B-P2 (moderately tolerant) and 841B-P3 (highly tolerant)]

Tift23D_2B_1-P1-P5. Contrary to it, the activity was enhanced by 3-folds (till 72h) under salt stress in the accession WSIL-P8. Though the activity declined by 96h, it was nearly 2-folds higher under stress conditions (till 144h) when compared to that of control seedlings grown without salt stress (Figure 4.14B).

4.2.3.3 Glutathione reductase (GR)

The native activity of GR was higher in the line Tift23D_2B_1-P1-P5 compared to WSIL-P8. The activity of GR increased (almost 2-folds) till 120h under salt stress but declined thereafter. In the susceptible line WSIL-P8, a gradual increase (3-4-folds) in GR activity was noticed till 96h under salt stress. It declined by 120h but displayed 6-fold increase in the activity at 144h (Figure 4.14C).

4.2.3.4 Glutathione-S-transferase (GST)

Activity of GST decreased under salt stress marginally till 144h in the line Tift23D_2B_1-P1-P5. On the other hand, the activity increased by 3-folds under salt stress in the line WSIL-P8 at 48h. It declined slightly by 96h but picked up by 3-folds again by 120h (Figure 4.14D).

4.2.3.5 Reduced Glutathione (GSH) content

The line Tift 23D_2B_1-P1-P5 showed an increase in GSH content till 72h under stress but thereafter the level of glutathione declined drastically (Figure 4.14E). There was a gradual increase in GSH content of WSIL-P8 till 48h under salt stress. The content decreased slightly by 72h but rose sharply by 96h (5.0moles of GSH compared to 2.5moles of GSH/g fwt. in control seedlings). Though the content of GSH declined by 120h, it increased again by 144h in WSIL-P8 (Figure 4.14E).

4.2.3.6 Lipid Peroxidation

Significant changes in lipid peroxidation were not noticed till 72h under salt stress in the tolerant line Tift 23D_2B_1-P1-P5 The MDA content rather fluctuated thereafter. The native levels of MDA were lower in the susceptible line WSIL-P8 compared to Tift 23D_2B_1-P1-P5. Lipid peroxidation in the sensitive line WSIL-P8 increased 5-folds by 48h, and then declined. The MDA levels could not be detected by 120h but it rose by 5-folds by 144h (Figure 4.14F).

Figure 4.14. Changes in antioxidative enzyme activity during short-term salt stress at 150mM NaCl in parental lines of pearl millet, WSIL-P8 (salt-sensitive) and Tift $23D_2B_1$-P1-P5 (highly salt-tolerant)
[(A) Catalase, (B) Superoxide dismutase]

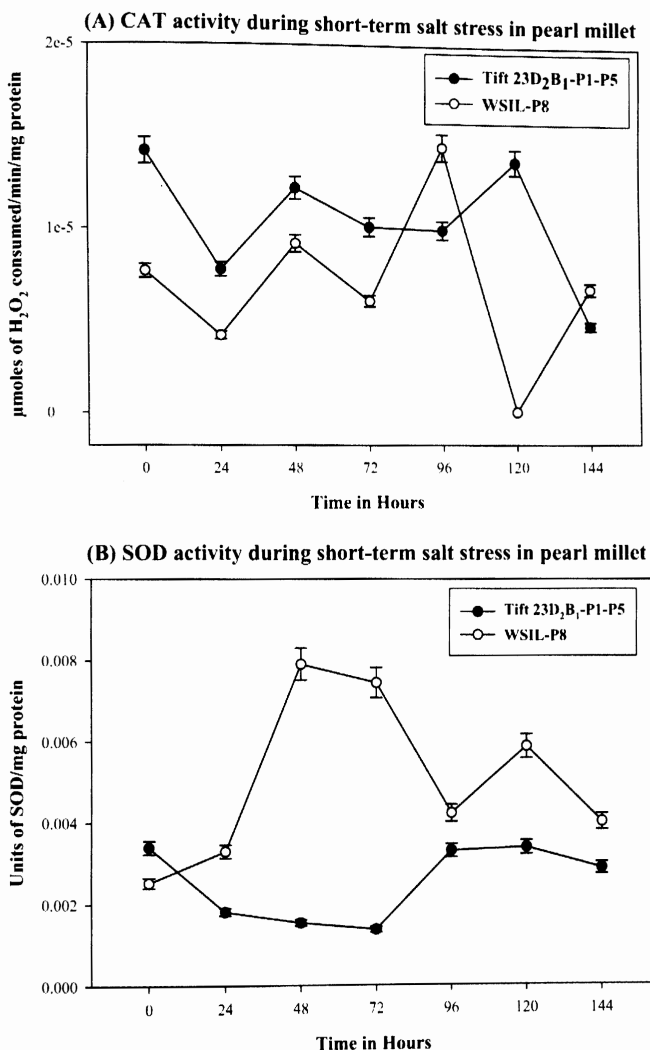
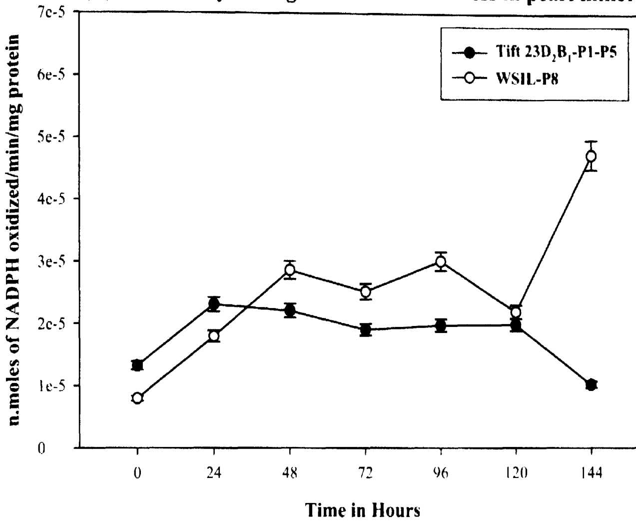


Figure 4.14. Changes in antioxidative enzyme activity during short-term salt stress at 150mM NaCl in parental lines of pearl millet, WSIL-P8 (salt-sensitive) and Tift 23D$_2$B$_1$-P1-P5 (highly salt-tolerant)
[(C) Glutathione reductase, (D) Glutathione-S-transferase]



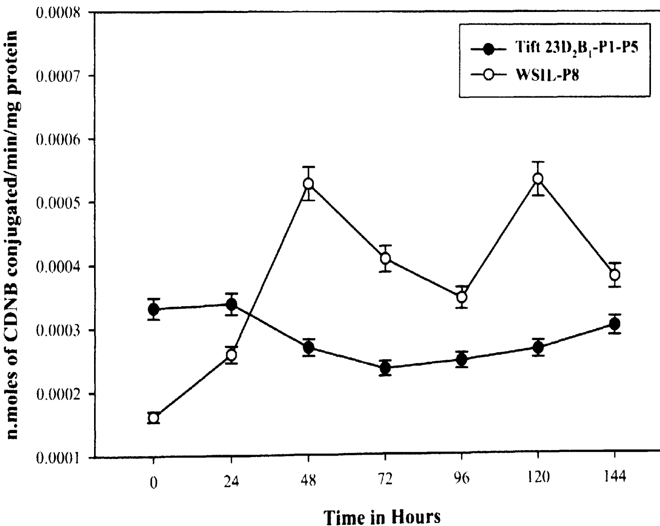
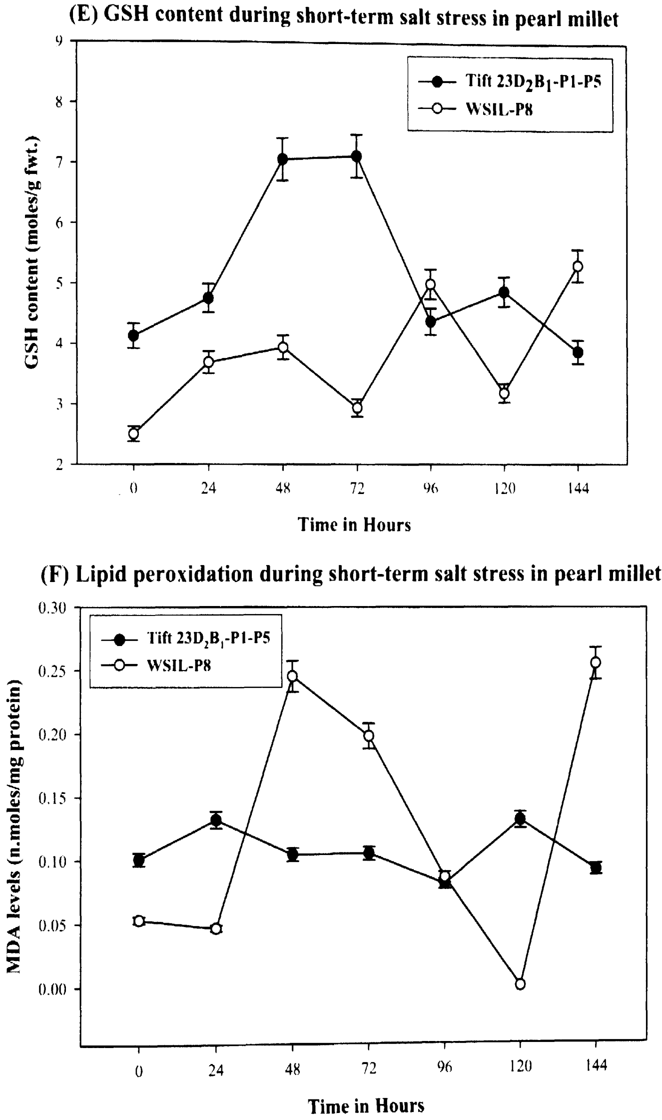


Figure 4.14. Changes in (E) Reduced Glutathione (GSH) and (F) Lipid peroxidation during short-term salt stress at 150mM NaCl in parental lines of pearl millet, WSIL-P8 (salt-sensitive) and Tift $23D_2B_1$-P1-P5 (highly salt-tolerant)



4.2.4 Isoenzyme profiles of five representative accessions of pearl millet

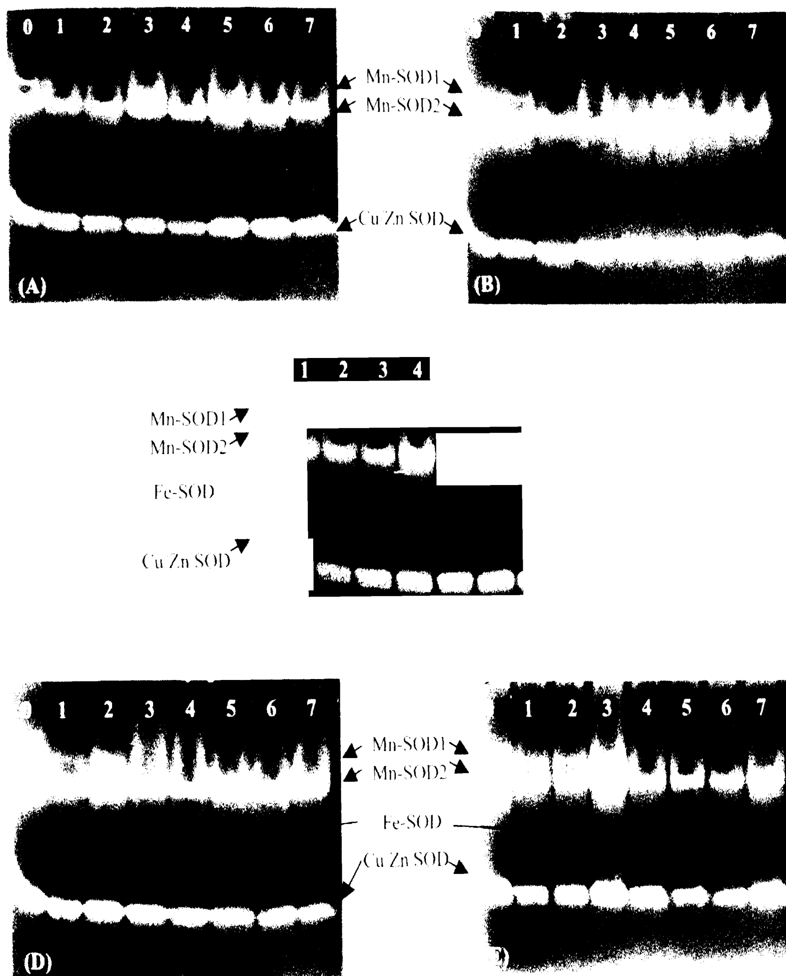
The activities of catalase (CAT) and ascorbate peroxidase (APX) were too low to be detected on native PAGE gels. However, SOD profiles were generated on native PAGE. Differences in the activities of Mn-SOD isoforms 1 and 2 and Cu/Zn-SOD were also noticed with increased exposure to salt in all of the lines (Plate 2). A total of three isoforms of SOD were detected of which two were Mn-SODs and one was a Cu-Zn SOD. The activity of the Cu-Zn SOD increased in all the lines under NaCl stress. However, the Mn-SOD isoform 1 displayed differential expression in sensitive, moderately tolerant and highly tolerant lines. In the salt-sensitive lines WSIL-P8 and ICMB 90111, activity of Mn-SOD1 was low all throughout the salinity treatment, while in the moderately tolerant line 863B-P2, and tolerant lines Tift $23D_2B_1$-P1-P5 and 841B-P3, this isoform disappeared after 1d, 2d and 3d, respectively. The second isoform Mn-SOD2 showed a gradual increase in activity with time in the sensitive lines all throughout the salinity stress treatment, whereas in the moderately tolerant and tolerant lines it increased with increased duration of exposure to salinity. Low activity of Fe-SOD was observed in seedlings of moderately tolerant and tolerant lines after 24h salt stress. However, no Fe-SOD isoform was observed in the sensitive seedlings exposed to 150mM NaCl. Fe-SOD bands were faint in intensity in the tolerant lines.

4.3 Protein profiles at short-term salt stress in seedlings of pearl millet

4.3.1 Short-term salt stress at 0mM, 75mM and 150mM NaCl for 0h, 24h, 40h & 72h in four accessions of pearl millet

Protein profiles in salt tolerant (P310-17Bk, 841B-P3, Tift $23D_2B_1$-P1-P5) and sensitive seedlings (81B-P6) of pearl millet are shown in Plate 3. Differences were noticed in the protein profiles with increasing salinity in short-term treatments (24h, 40h and 72h). At 24h salt treatment, tolerant lines 841B-P3 and P310-17Bk behaved differently with respect to a 180kDa protein. While in 841B-P3, there was an increase in the band intensity of this protein with increasing salinity, the same protein band decreased in intensity with increasing salt levels in the line P310-17B. A similar observation was recorded for a 54kDa protein in the same two lines after 72 h of saline treatments. There

Plate 2. Isoenzyme patterns of superoxide dismutase in pearl millet lines (A) WSIL-P8, (B) ICMB 90111, (C) 863B-P2, (D) Tift $23D_2B_1$-P1-P5 and (E) 841B-P3 at 150mM NaCl in short-term salinity treatments



[0= 0 days (control), 1= 1 day, 2= 2 days, 3= 3 days, 4= 4 days, 5= 5 days, 6= 6 days and 7= 7 days]

were significant differences among susceptible and tolerant lines with respect to a 120kDa protein, which was absent in the line 81B-P6 (salt sensitive), but present in all three tolerant lines *i.e.*, Tift $23D_2B_1$-P1-P5, 841B-P3 and P310-17Bk at all the three salinity levels.

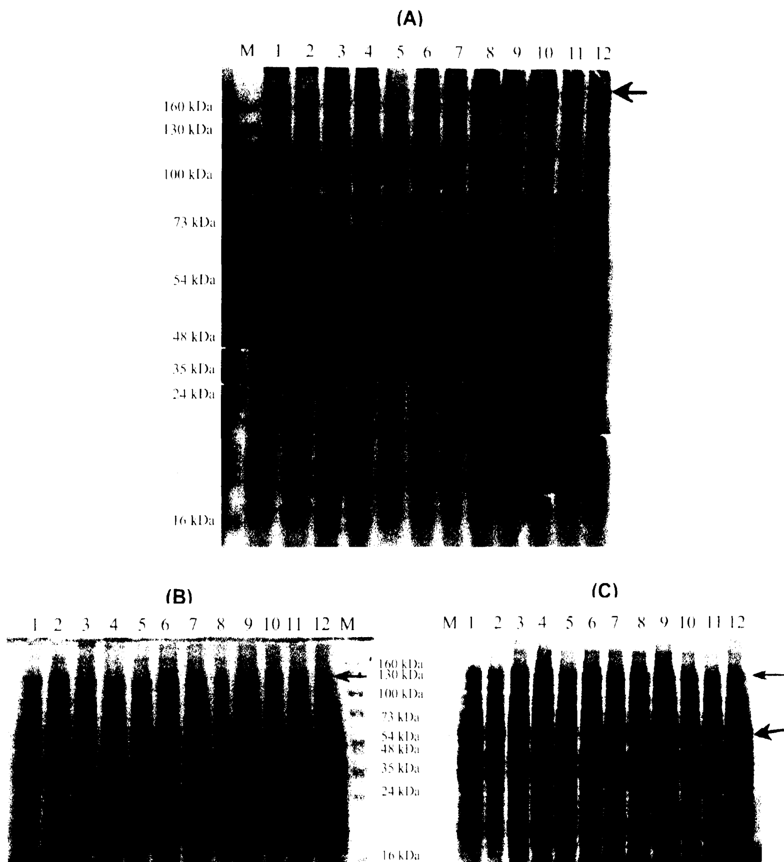
4.3.2 Protein content of seedlings at short-term salt stress

A general decrease in protein content was observed in the sensitive lines WSIL-P8 after exposure to salt stress. But in the other susceptible line ICMB 90111, there was a transient increase by 24h, which declined later drastically (Table 4.11). The protein content doubled by 48h in the line 863B-P2, declined slightly there after and remained more or less stable till 144h. Protein content in the line Tift $23D_2B_1$-P1-P5 was 14.55mg/g dry tissue under control conditions. It enhanced to 32.62 mg by 72h, but declined to 15.17mg/g dry tissue by 96h. In the line 841B-P3, protein content declined till 72h, but increased considerably by 96h under salt stress. The protein content however declined thereafter and reached a value almost equal to that of control at 144h (Table 4.11).

4.3.3 Short term salt stress of five accessions of pearl millet at 150mM NaCl for 0h, 24h, 48h, 72h, 96h, 120h, 144h and 168h

Protein profiles as seen in Plate No. 4 were found supportive of the total protein data (section 4.4.2) of the pearl millet lines exposed to salt stress for short-durations. Though quantitative changes were noticed in the protein, not many differences at the qualitative level were recorded in different lines of pearl millet under salt stress excepting 25kDa, 27kDa and 54kDa protein bands especially in the moderately tolerant and highly tolerant lines under salt stress. The 25kDa protein band was not noticed in either of the sensitive lines WSIL-P8 and ICMB 90111, while the 27kDa and the 54kDa bands showed increased intensity in the highly tolerant and moderately tolerant lines compared to the sensitive lines under salt stress (indicated by arrows in Plate 4). In addition, about 15 kDa and 18 kDa bands were also noticed especially in the tolerant lines.

Plate 3. Protein profiles of 0mM, 75mM and 150mM NaCl treated seedlings of pearl millet (lanes 1-3: Tift $23D_2B_1$-P1-P5; lanes 4-6: 81B-P6; lanes 7-9: 841B-P3; lanes 10-12: P310-17-Bk; 0mM, 75mM and 150mM NaCl treated respectively) for (A) 24 h, (B) 48 h and (C) 72 h treatments



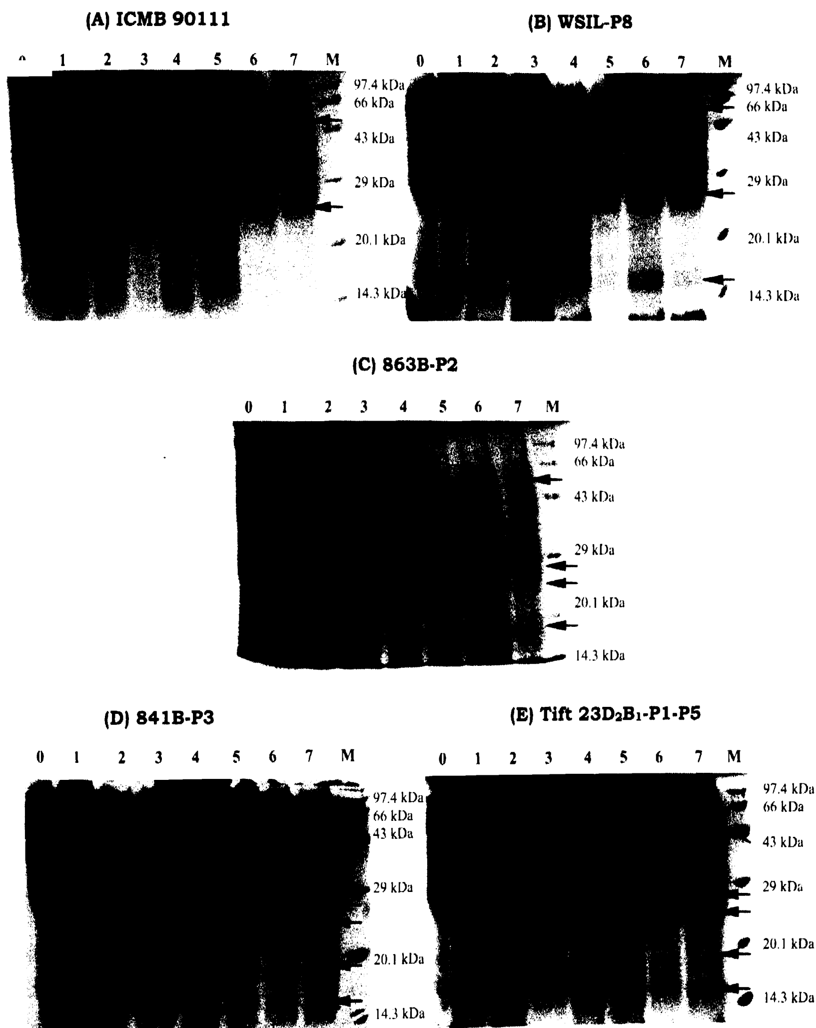
[Note: Thick arrows = increase in band intensity in 7, 8, 9 and decrease in band intensity in 10, 11 and 12 with increased salinity treatment
Thin arrows = absence of 120kDa bands in 4,5,6 (81B-P6) and its presence in rest of the lines]

Table 4.11. Total Protein content (mg/g dry wt.) in pearl millet lines subjected to short-term salt stress at 150mM NaCl

Time in Hours	Total Protein (mg/g dry wt.)				
	WSIL-P8	ICMB 90111	863B-P2	Tift $23D_2B_1$-P1-P5	841B-P3
0	16.13 (±0.5)	16.09 (±0.4)	9.96 (±0.1)	14.55 (±0.3)	10.13 (±0.2)
24	13.15 (±0.1)	20.89 (±0.1)	11.31 (±0.5)	19.50 (±0.1)	10.17 (±0.5)
48	11.98 (±0.3)	17.34 (±0.1)	20.63 (±0.2)	28.15 (±0.3)	7.29 (±0.5)
72	13.62 (±0.1)	9.19 (±0.3)	14.67 (±0.5)	32.62 (±0.5)	7.38 (±0.2)
96	8.54 (±0.2)	9.40 (±0.2)	15.54 (±0.1)	15.17 (±0.2)	15.03 (±0.2)
120	7.33 (±0.5)	10.13 (±0.2)	12.75 (±0.2)	14.02 (±0.4)	13.44 (±0.1)
144	8.15 (±0.2)	11.76 (±0.5)	17.75 (±0.4)	14.64 (±0.1)	9.69 (±0.2)

*** Data represent mean of two replicates**
*** Values in the parenthesis indicate the standard errors**
*** P value < 0.05**

Plate 4. Protein profiles of five accessions of pearl millet at 150mM NaCl for short-term salt stress for 0h (0), 24h (1), 48h (2), 72h (3), 96h (4), 120h (5), 144h (6) and 168h (7). [(A) ICMB 90111, (B) WSIL-P8, (C) 863B-P2, (D) 841B-P3 and (E) Tift $23D_2B_1$-P1-P5]



[Note: Presence/absence of arrows = presence/absence of 15kDa, 18kDa, 25kDa, 27kDa and 54kDa bands]

4.4 Effect of GA_3 and $CaCl_2$ on response to salinity stress in pearl millet seedlings

Addition of $CaCl_2$ and GA_3 slightly alleviated the adverse effects of salt stress on germination and early seedling growth of two inbred lines of pearl millet, ICMB 90111, a sensitive line and 841B-P3, a tolerant line. Because the two lines tested have different degrees of salt tolerance, one of the primary objectives was to determine whether these lines respond better to the combination of salinity with $CaCl_2$ or GA_3 or both, with respect to seed germination, growth and proline content. The frequency of seed germination of the sensitive line improved slightly in 150mM NaCl in 841B-P3 when treated with GA_3 and also with GA_3 plus $CaCl_2$. The germination percentage of the sensitive line ICMB 90111 also improved at salt concentrations of 100mM and 150mM NaCl when treated with GA_3 and $CaCl_2$ individually and in combination (Fig. 4.15). Both lines were found to be highly responsive to exogenous GA_3 and exhibited significant differences in lengths and fresh weights of their shoots and roots in salt media amended with GA_3 (Figures 4.16 and 4.17). Although the growth of seedlings declined with an increase in NaCl, application of $CaCl_2$ and GA_3 or both appear to have alleviated the salt stress effects on both the lines (Figures 4.15, 4.16 and Plates 5 and 6). A decline in fresh weights of shoots and roots was noticed in these lines in presence of salt stress (Fig. 4.16). In presence of $CaCl_2$ and GA_3 individually and together, the salinity-sensitive line ICMB 90111 not only germinated and grew well, but also accumulated proline significantly when compared to the non-amended controls. The proline content in the tolerant line 841B-P3 was 6 times higher than to its control value in the presence of $CaCl_2$ and GA_3 (Fig. 4.18).

4.5 Development of molecular markers

4.5.1 Data mining and primer designing for TRAP markers

The NCBI database (www.ncbi.nlm.nih.gov) proved very rapid and useful to retrieve sequences of our interest. Maize ESTs for GR6, GR8 and GR13 (glutathione reductase gene), having accession numbers CN844501, CK787292 and CF349127 were obtained by giving the key words '*Zea mays* and

Figure 4.15. Effect of $CaCl_2$ and GA_3 on the germination percentage of a set of pearl millet inbred lines [ICMB 90111and 841B-P3 (sensitive and tolerant to salt stress, respectively)] under 75mM, 100mM and 150mM NaCl stress as compared to their respective 0mM NaCl controls

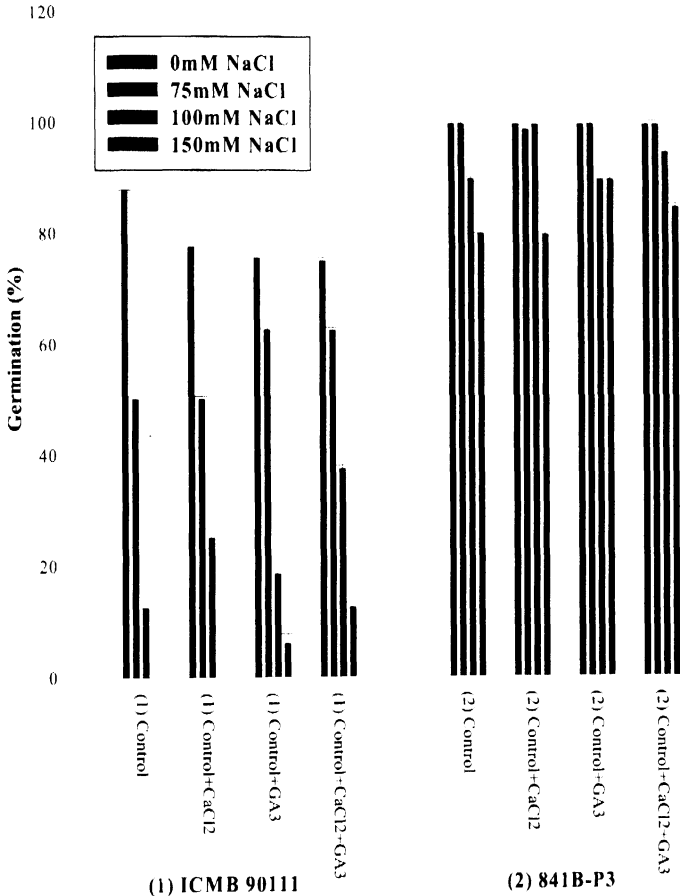
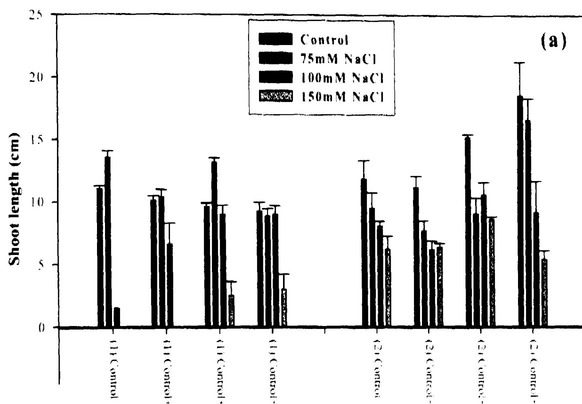


Figure 4.16. Effect of $CaCl_2$ and GA_3 on the (a) shoot length and (b) root length of a set of pearl millet inbred lines [ICMB 90111and 841B-P3 (sensitive and tolerant to salt stress, respectively)] under 75mM, 100mM and 150mM NaCl stress as compared to their respective 0mM NaCl controls



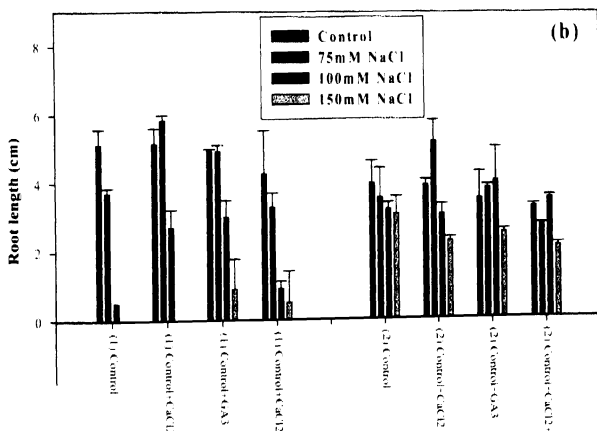
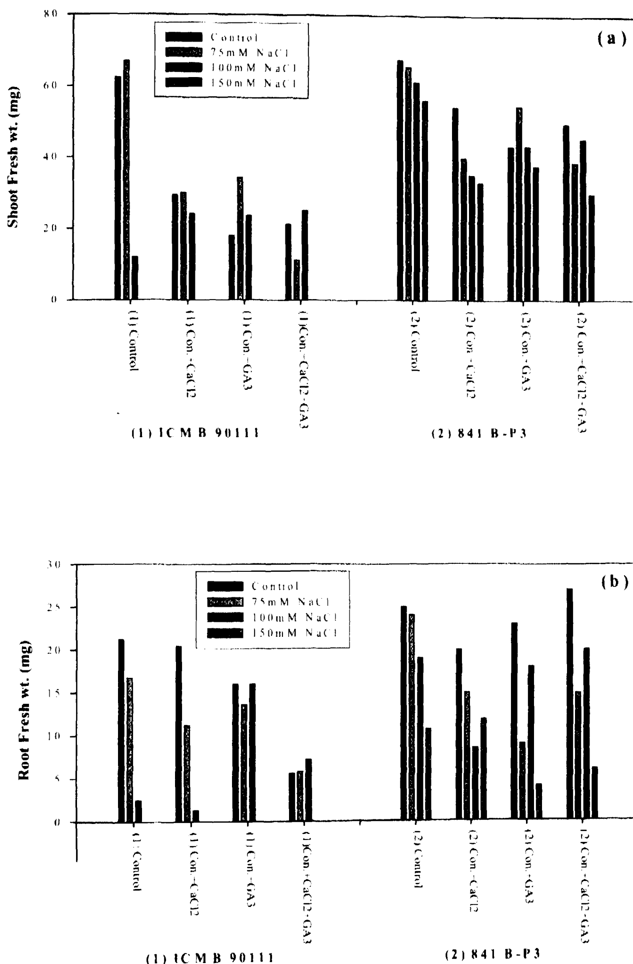
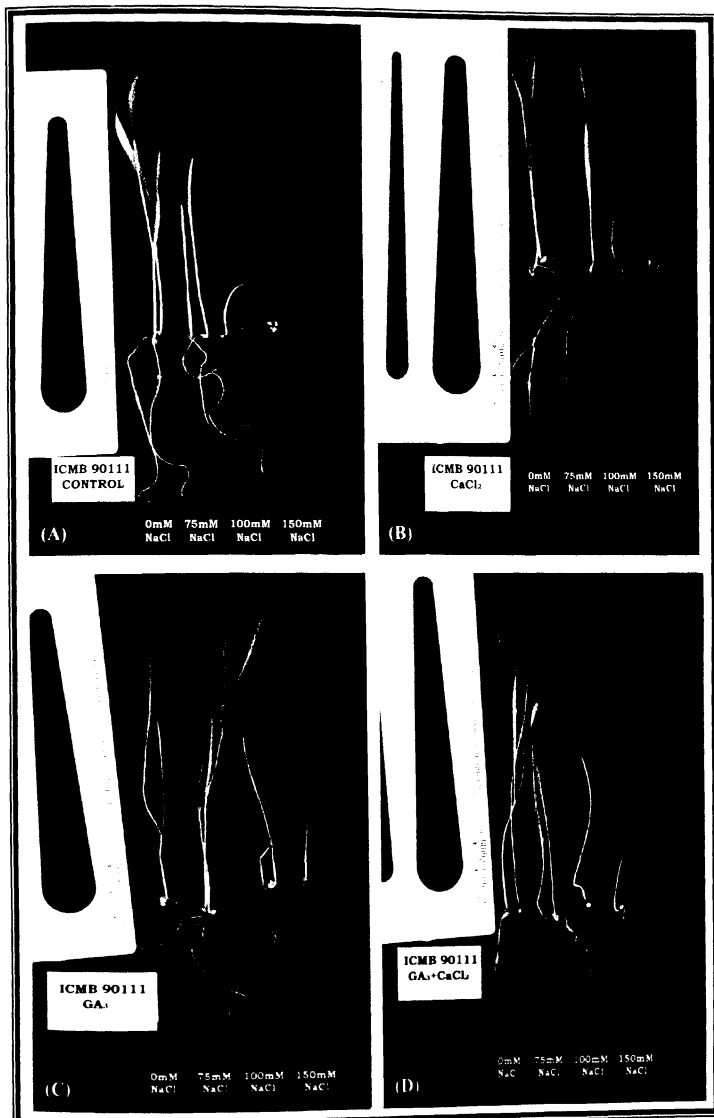


Figure 4.17. Effect of $CaCl_2$ and GA_3 on the (a) shoot fresh weights and (b) root fresh weights, of a set of pearl millet inbred lines [ICMB 90111and 841B-P3 (sensitive and tolerant to salt stress, respectively)] under 75mM, 100mM and 150mM NaCl stress as compared to their respective 0mM NaCl controls



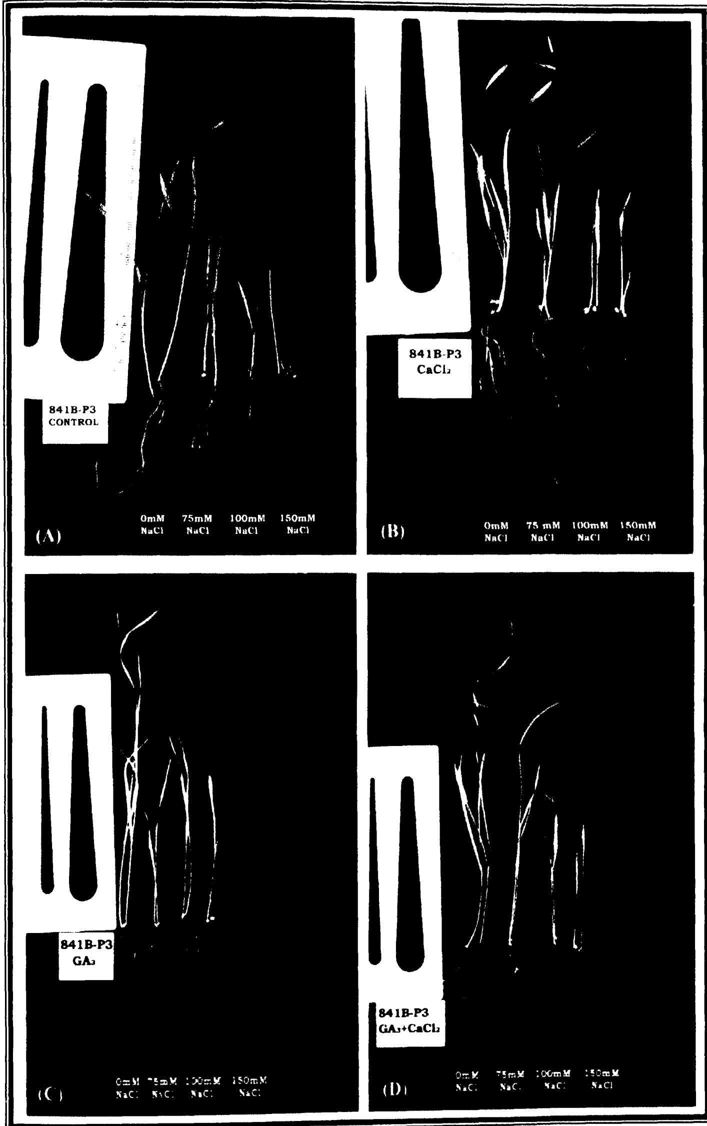
[Data represent mean of 10 seedlings measured for the traits]

Plate 5. Effect of $CaCl_2$ (B), GA_3 (C) and both [GA_3+$CaCl_2$] (D) as compared to the control (A) in 10-day-old seedlings of pearl millet line ICMB 90111 grown in 0mM, 75mM, 100mM and 150mM NaCl media



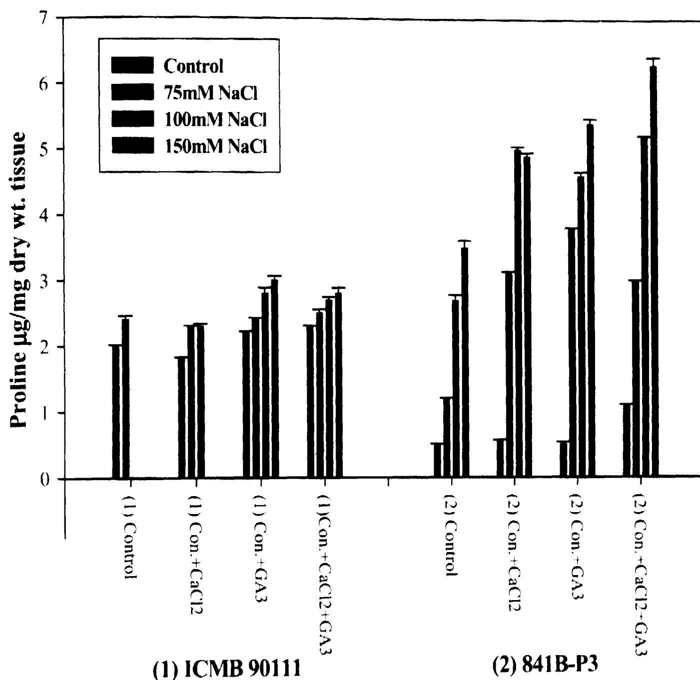
[Note: Germination improves at 100mM and 150mM NaCl in (B), (C) and (D) mpared to their respective controls (A).]

Plate 6. Effect of $CaCl_2$ (B), GA_3 (C) and both [GA_3+$CaCl_2$] (D) as compared to the control (A) in 10-day-old seedlings of pearl millet line 841B-P3 grown in 0mM, 75mM, 100mM and 150mM NaCl media



[Note: There is slight improvement of shoot length at 100mM and 150mM NaCl in (B), (C) and (D) as compared to their respective controls (A).]

Figure 4.18. Effect of $CaCl_2$ and GA_3 on the proline content of a set of pearl millet inbred lines [ICMB 90111 and 841B-P3 (sensitive and tolerant to salt stress, respectively)] under 75mM, 100mM and 150mM NaCl stress as compared to their respective 0mM NaCl controls



glutathione reductase' in the search tool available in the NCBI database. The accession numbers of maize EST for SOD2, SOD3, SOD5, SOD6 and SOD7 (superoxide dismutase genes) were CN844460, CK849972, CK786884, CK786803 and BQ619487, respectively. The accession number CN845408 of a pearl millet sequence for a P5CS gene was also obtained directly from the NCBI database using the search tool. The sequences were retrieved in FASTA format for ease of handling to design primers.

GRAMENE BLAST (www.gramene.org/multi/blastview) search results and the selected sequences for alignment from barley, millet, rice and sorghum are given in Appendix I. None of the fixed primers designed fell in the interface of the exon and intron when checked using the 'ORF finder' in NCBI database. The lists of the fixed and arbitrary primers designed are given in Tables 3.5 and 3.6, respectively, in Chapter 3.

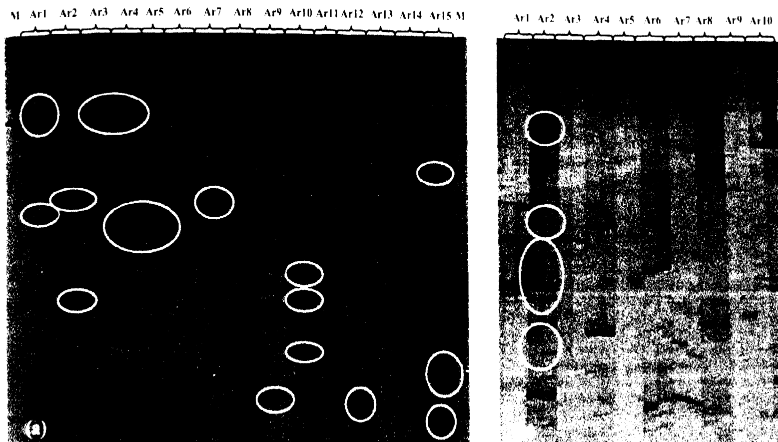
4.5.2 Optimization of TRAP technique

In the present study, DNA concentration was optimized to 5ng per 5μl PCR reaction. Initial screening of the parental lines (Tift $23D_2B_1$-P1-P5 and WSIL-P8) to identify polymorphic TRAP markers was performed using combinations of some selected fixed primers with all of the 15 arbitrary primers and TRAP marker profiles were generated in silver-stained 6.5% polyacrylamide gels as shown in Plate 7. For clear detection of the multiple bands generated by TRAP-PCRs, a 68-well comb was used for PAGE profiling.

4.5.3 TRAP marker analysis

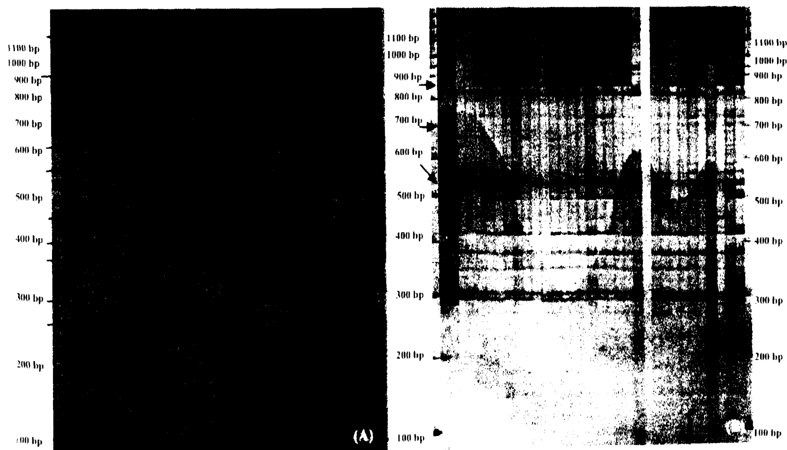
In total, 180 primer combinations (12 fixed primers comprised of 10 forward and 2 reverse primers in combination with 15 arbitrary primers) were checked for polymorphism among the two mapping population parents. Fifteen PCR reactions were carried with each of the fixed primers, as there were 15 arbitrary primers. Each PCR reaction generated about 15-25 potential TRAP marker bands, which significantly varied in band intensity. The size of most of the fragments ranged from 130bp to 1590bp, all the bands from each PCR were highly reproducible except for a few weak bands in some combinations. On an

Plate 7. Optimization of PAGE gel profiles for detecting TRAP polymorphisms between Tift $23D_2B_1P1$-P5 (parent A) and WSIL-P8 (parent B) with (a) P5CSF and (b) GR6F fixed TRAP primers in combination with arbitrary TRAP primers Ar1 to Ar15



[Note: Encircled = A few possible polymorphic bands that can be scored across the mapping population]

Plate 8. Optimization of TRAP technique among the mapping population progenies of Tift $23D_2B_1$-P5 × WSIL-P8 with TRAP primer combinations (A) GR6F-Ar2 and (B) SOD3F-Ar4



average, each TRAP primer pair was able to generate 6 polymorphic TRAP markers that were clear and easily scorable when visualized using PAGE and silver staining procedures (Plate 7). Based on the clarity and intensity of the bands and their reproducibility, 11 fixed-arbitrary primer combinations were selected to screen for the same markers in the $F_{2:4}$ mapping population available. DNA concentrations for the TRAP-PCR and the PAGE technique were optimized for the mapping population before generating the final marker profiles (Plate 8).

Eleven TRAP PCR reactions that employed fixed EST-based primers in combination with arbitrary primers generated 68 easily scorable polymorphic bands (Table 4.12). These 68 markers were scored on the $F_{2:4}$ mapping population of Tift $23D_2B_1$-P1-P5 × WSIL-P8. These marker polymorphisms were highly reproducible among the parents as well as the mapping population progenies. Plates 9, 10 and 11 show parental polymorphism in the TRAP markers selected for screening of the mapping population progenies. Plates 12, 13, 14, 15 and 16 show TRAP marker profiles for the segregating mapping population progenies. The TRAP score sheet is represented in Appendix III.

When these TRAP markers were analyzed for segregation distortion using a chi-square test (Table 4.13), it was found that segregation patterns of 22 TRAP markers were not significantly distorted, while 21 TRAP markers showed distortion at 1% level of significance and eight were significant at 5% level. For the ratio 1:3, segregation patterns of 7 TRAP markers were not significantly distorted, 4 showed distortion at 1% level of significance and 6 were significant at 5% level. They were also tested for segregation into 1:1 ratio and 19 TRAP markers were found segregating with no significant distortion. Hence, 22 and 7 out of the 68 TRAP markers segregated according to the expected Mendelian ratio of 3:1 or 1:3, respectively, while 19 TRAP markers segregated according to the expected ratio of 1:1 (Table 4.13).

4.5.4 Construction of linkage map

Out of 68 polymorphic TRAP markers identified, 4 pairs of markers viz. S2A7.630 and S2A7.610, S3A4.530 and S3A4.510, PA15.290 and PA15.280

Table 4.12. Details of TRAP markers developed in this study

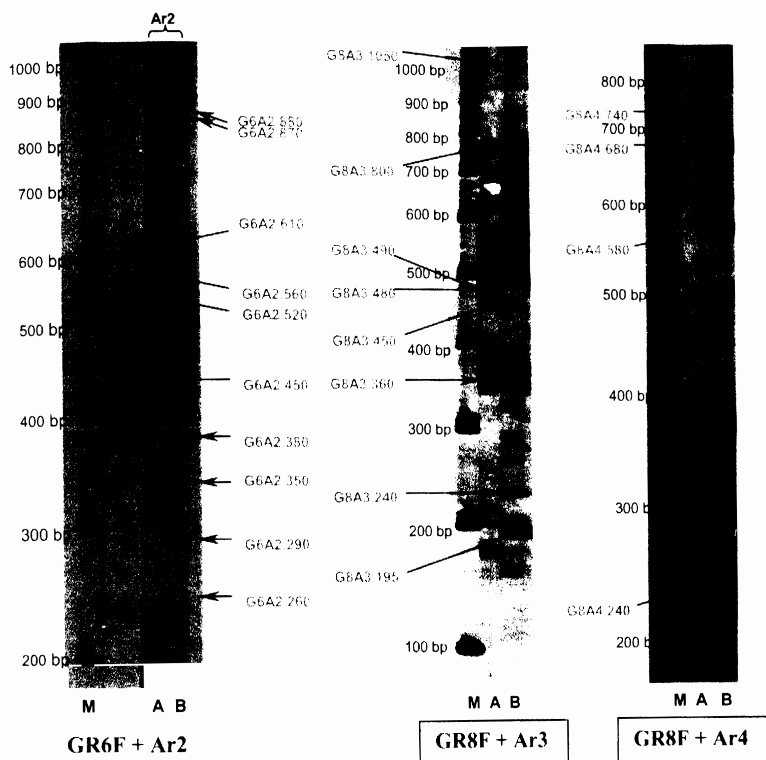
S.No	Marker Name	Tift $23D_2B_1$-P1-P5 = Parent A	WSIL-P8 = Parent B	Size (bp) Approx.	Primer Combinations	
					Fixed Primer	Arbitrary Primer
1	G6A2.880	0 (A)	1 (C)	880	GR6F	Ar2
2	G6A2.870	0 (A)	1 (C)	870		
3	G6A2.610	1 (D)	0 (B)	610		
4	G6A2.560	1 (D)	0 (B)	560		
5	G6A2.520	1 (D)	0 (B)	520		
6	G6A2.450	1 (D)	0 (B)	450		
7	G6A2.380	0 (A)	1 (C)	380		
8	G6A2.350	0 (A)	1 (C)	350		
9	G6A2.290	0 (A)	1 (C)	290		
10	G6A2.260	0 (A)	1 (C)	260		
11	G8A3.1050	1 (D)	0 (B)	1050	GR8F	Ar3
12	G8A3.490	1 (D)	0 (B)	490		
13	G8A3480	1 (D)	0 (B)	480		
14	G8A3.450	0 (A)	1(C)	450		
15	G8A3.360	1 (D)	0 (B)	360		
16	G8A3.240	0 (A)	1 (C)	240		
17	G8A3.195	0 (A)	1 (C)	195		
18	G8A4.740	1 (D)	0 (B)	740	GR8F	Ar4
19	G8A4.680	0 (A)	1 (C)	680		
20	G8A4.580	0 (A)	1 (C)	580		
21	G8A4.240	0 (A)	1 (C)	240		
22	S2A7.1500	1 (D)	0 (B)	1500	SOD7F	Ar7
23	S2A7.980	0 (A)	1 (C)	980		
24	S2A7.880	1 (D)	0 (B)	880		
25	S2A7.690	1 (D)	0 (B)	690		
26	S2A7.630	1 (D)	0 (B)	630		
27	S2A7.610	0 (A)	1 (C)	610		
28	S2A7.120	0 (A)	1 (C)	120		
29	S3A4.1550	1 (D)	0 (B)	1550	SOD3F	Ar4
30	S3A4.950	0 (A)	1 (C)	950		
31	S3A4.890	0 (A)	1 (C)	890		
32	S3A4.660	1 (D)	0 (B)	660		
33	S3A4.530	0 (A)	1 (C)	530		
34	S3A4.510	1 (D)	0 (B)	510		
35	S3A11.1590	1 (D)	0 (B)	1590	SOD3F	Ar11
36	S3A11.850	0 (A)	1 (C)	850		
37	S3A11.760	0 (A)	1 (C)	760		
38	S3A11.490	1 (D)	0 (B)	490		
39	S3A11.450	0 (A)	1 (C)	450		
40	S5A8.690	1 (D)	0 (B)	690	SOD5MF	Ar8
41	S5A8.490	0 (A)	1 (C)	490		
42	S5A8.340	0 (A)	1 (C)	340		
43	S5A8.260	0 (A)	1 (C)	260		

Table 4.12 contd.

S.No	Marker Name	Tift $23D_2B_1$-P1-P5 = Parent A	WSIL-P8 = Parent B	Size (bp) Approx.	Primer Combinations	
					Fixed Primer	Arbitrary Primer
44	S5A9.700	1 (D)	0 (B)	700	SOD5MF	Ar9
45	S5A9.510	0 (A)	1 (C)	510		
46	S5A9.410	0 (A)	1 (C)	410		
47	S5A9.210	1 (D)	0 (B)	210		
48	S5A9.110	1 (D)	0 (B)	110		
49	PA1.1040	0 (A)	1 (C)	1040	P5CSF	Ar1
50	PA1.990	0 (A)	1 (C)	990		
51	PA1.910	0 (A)	1 (C)	910		
52	PA1.805	1 (D)	0 (B)	805		
53	PA1.590	0 (A)	1 (C)	590		
54	PA1.550	1 (D)	0 (B)	550		
55	PA1.520	0 (A)	1 (C)	520		
56	PA1.380	1 (D)	0 (B)	380		
57	PA1.220	1 (D)	0 (B)	220		
58	PA8.1050	0 (A)	1 (C)	1050	P5CSF	Ar8
59	PA8.990	0 (A)	1 (C)	990		
60	PA8.510	0 (A)	1 (C)	510		
61	PA8.130	1 (D)	0 (B)	130		
62	PA15.1050	0 (A)	1 (C)	1150	P5CSF	Ar15
63	PA15.550	1 (D)	0 (B)	550		
64	PA15.290	1 (D)	0 (B)	290		
65	PA15.280	0 (A)	1 (C)	280		
66	PA15.200	1 (D)	0 (B)	200		
67	PA15.165	0 (A)	1 (C)	165		
68	PA15.160	1 (D)	0 (B)	160		

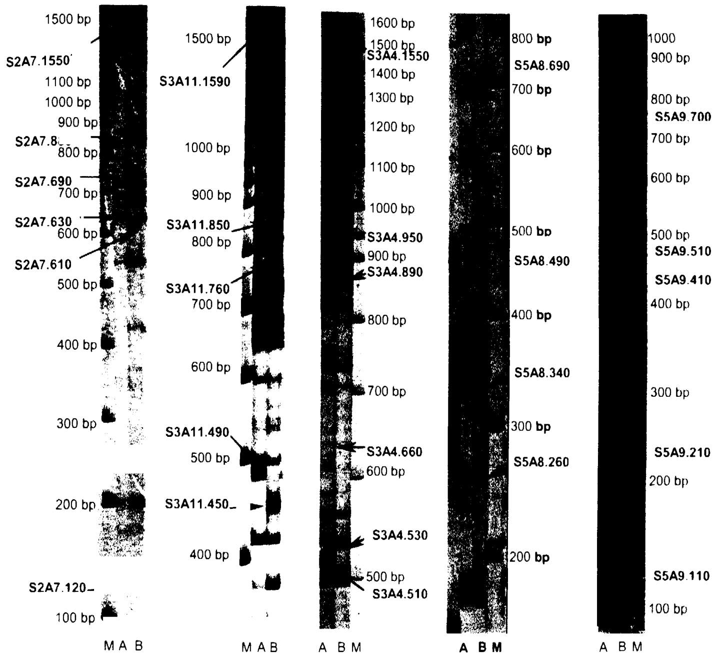
(Note: 1. Presence of band is represented as '1' and absence of band is represented as '0'.
2. 'A', 'B', 'C', and 'D' are the corresponding scores for bands in the mapping population.)

Plate 9. Glutathione reductase (GR) based TRAP polymorphisms in Tift 23D₂B₁P1-P5 (parent A) and WSIL-P8 (parent B)



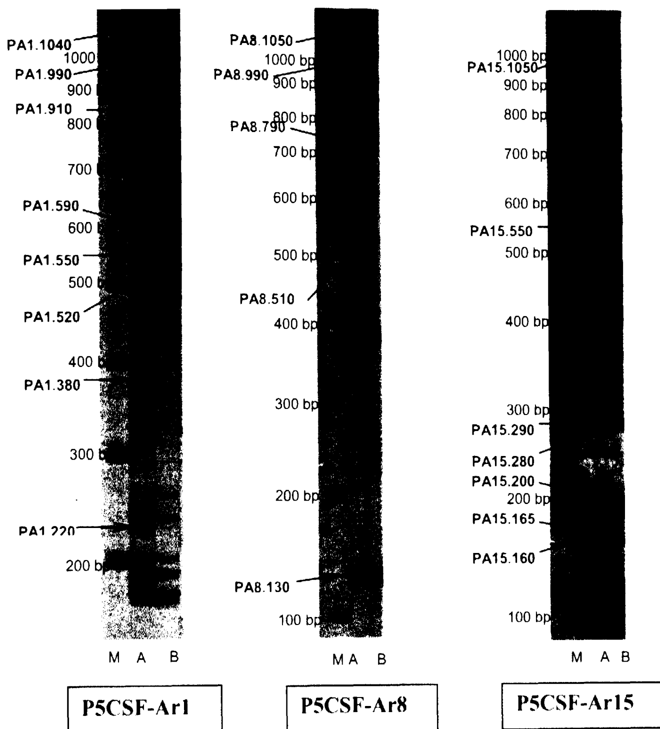
(M= 100 bp marker; A=Tift $23D_2B_1$-P1-P5; B=WSIL-P8)

Plate 10. Superoxide dismutase (SOD) based TRAP polymorphisms in Tift $23D_2B_1P1$-P5 (parent A) and WSIL-P8 (parent B)



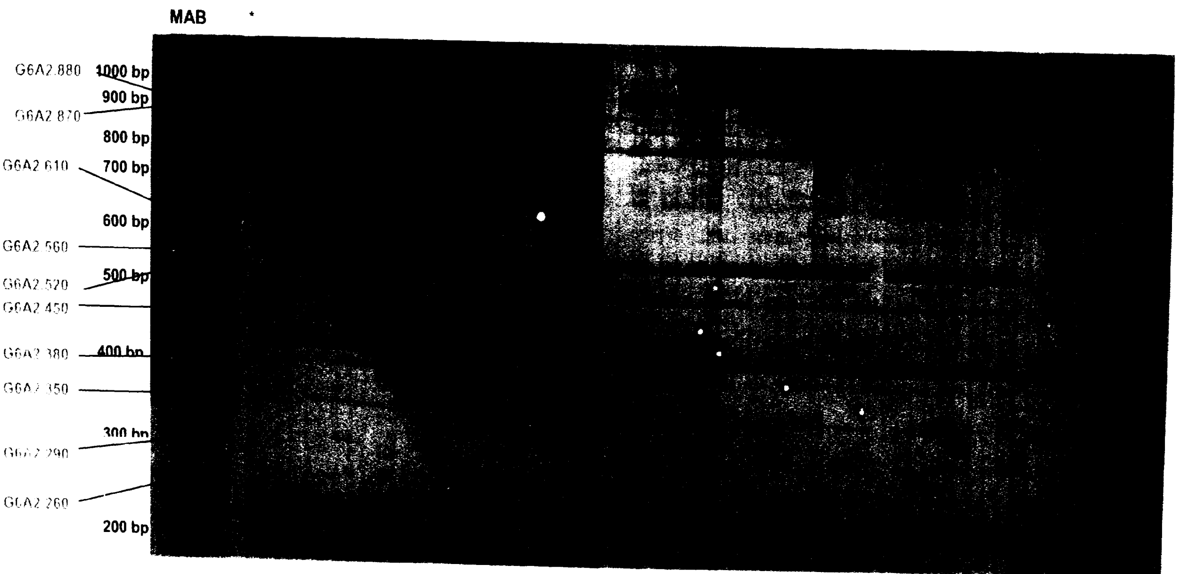
(M= 100 bp marker; A=Tift $23D_2B_1$-P1-P5; B=WSIL-P8)

Plate 11. Δ^1**-pyrroline-5-carboxylate synthetase (P5CS) based TRAP polymorphisms in Tift 23D$_2$B$_1$P1-P5 (parent A) and WSIL-P8 (parent B)**



(M= 100 bp marker; A=Tift 23 D$_2$B$_1$-P1-P5; B=WSIL-P8)

Plate 12. TRAP marker profiles seen among the mapping population progenies of Tift $23D_2B_1$-P1-P5 × WSIL-P8 with primer combination GR6F-Ar2

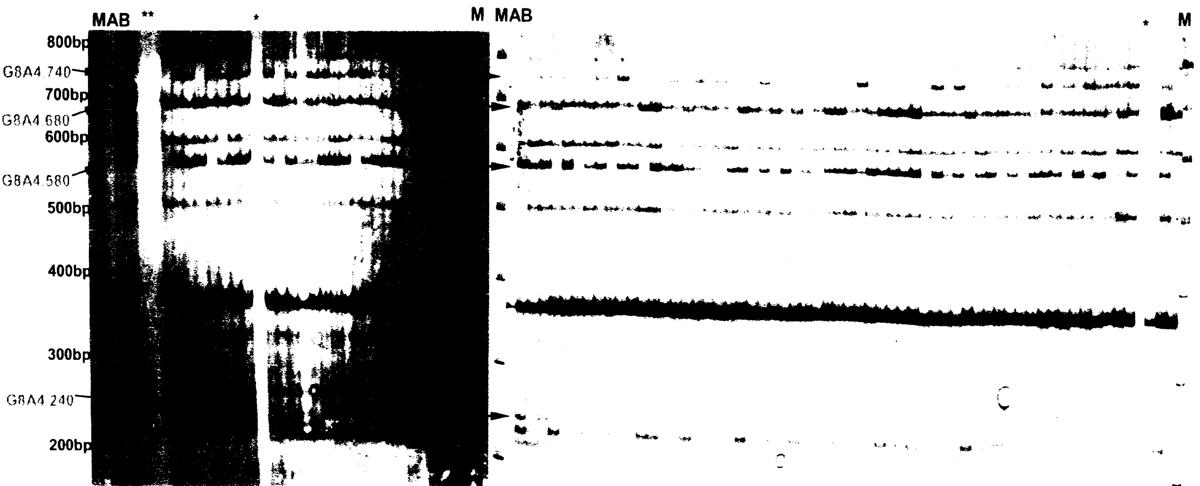


* = Blank wells

GR6F + Ar2

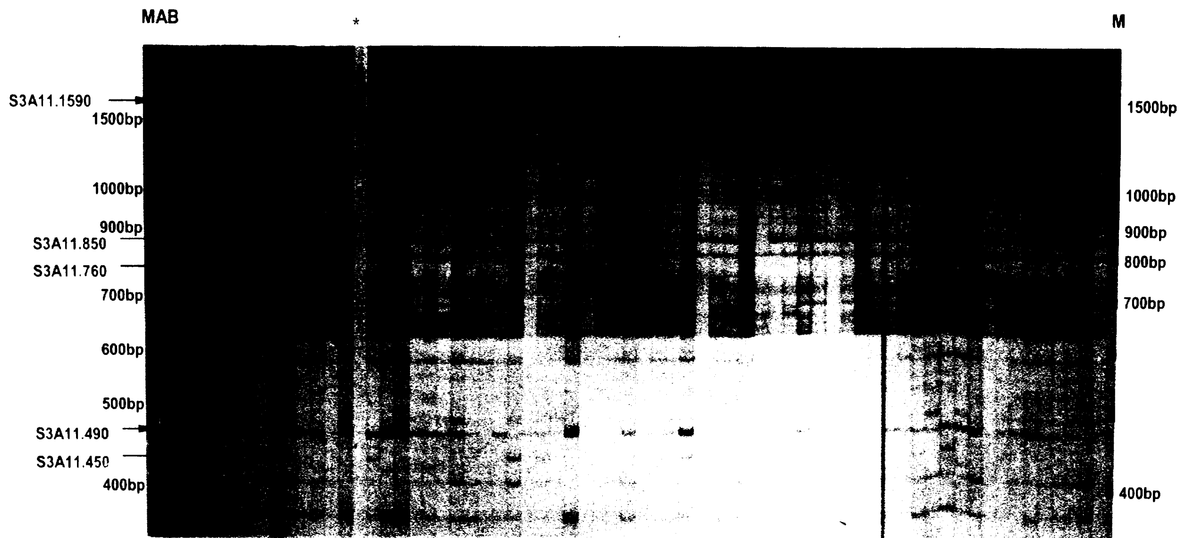
(M= 100 bp marker; A=Tift23 D_2B_1-P1-P5; B=WSIL-P8)

Plate 13. TRAP marker profiles seen among the mapping population progenies of Tift $23D_2B_1$-P1-P5 × WSIL-P8 with primer combination GR8F-Ar4



(M= 100 bp marker; A=Tift $23D_2B_1$-P1-P5; B=WSIL-P8)

Plate 15. TRAP marker profiles seen among the mapping population progenies of Tift $23D_2B_1$-P1-P5 × WSIL-P8 with primer combination SOD3F-Ar11

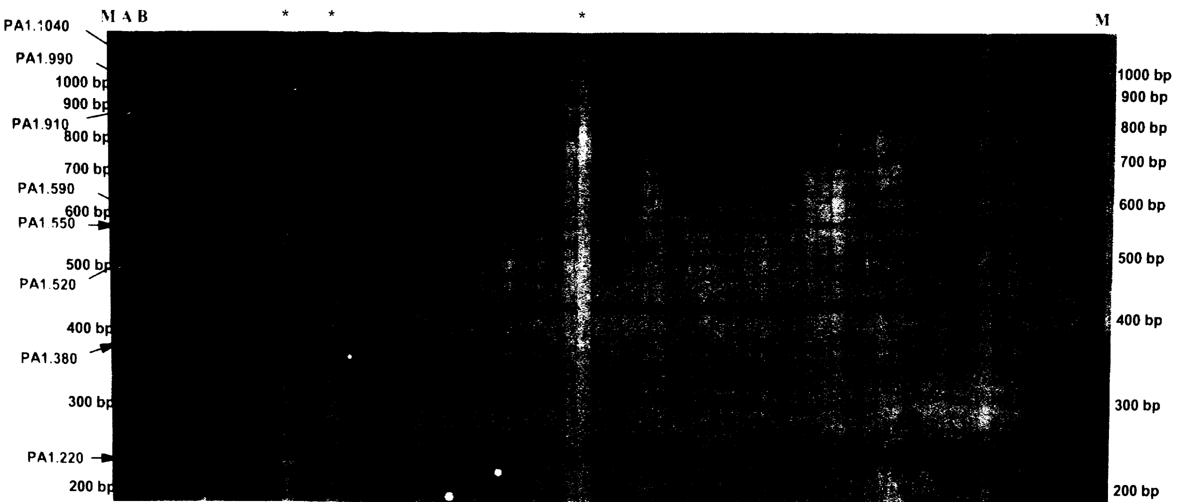


* = Blank wells

SOD3F + Ar11

(M= 100 bp marker; A=Tift $23D_2B_1$-P1-P5; B=WSIL-P8)

Plate 16. TRAP marker profiles seen among the mapping population progenies of Tift $23D_2B_1$-P1-P5 × WSIL-P8 with primer combination P5CSF-Ar1



(M= 100 bp marker; A=Tift $23D_2B_1$-P1-P5; B=WSIL-P8)

Table 4.13. Chi-square analysis of segregation distortion of TRAP markers

S.No.	Marker Loci	Presence (1s)	Absence (0s)	Chi Square values (Expected ratio 3:1 or 1:3)	Chi Square values (Expected ratio 1:1)
1	G6A2.260	50	46	26.89 **	0.17 ns
2	G6A2.290	49	48	31.01 **	0.01 ns
3	G6A2.350	35	62	6.35 *	7.52 **
4	G6A2.380	68	29	1.24 ns	15.68 **
5	G6A2.450	18	77	1.86 ns	36.64 **
6	G6A2.520	33	64	4.21 *	9.91 **
7	G6A2.560	79	16	3.37 ns	41.78 **
8	G6A2.610	62	35	6.35 *	7.52 **
9	G6A2.870	31	66	2.51 ns	12.63 **
10	G6A2.880	25	72	0.03 ns	22.77 **
11	G8A3.1050	49	42	21.72 **	0.54 ns
12	G8A3.195	73	23	0.06 ns	26.04 **
13	G8A3.240	47	44	26.47 **	0.10 ns
14	G8A3.360	66	31	2.51 ns	12.63 **
15	G8A3.450	40	55	14.82 **	2.37 ns
16	G8A3.480	54	43	19.33 **	1.25 ns
17	G8A3.490	59	37	9.39 **	5.04 *
18	G8A4.240	57	40	13.64 **	2.98 ns
19	G8A4.580	69	28	0.77 ns	17.33 **
20	G8A4.680	53	44	21.45 **	0.84 ns
21	G8A4.740	69	28	0.77 ns	17.33 **
22	S2A7.120	58	38	10.89 **	4.17 *
23	S2A7.1500	85	11	9.39 *	57.04 **
24	S2A7.610	55	40	14.82 **	2.37 ns
25	S2A7.630	84	11	9.13 **	56.09 **
26	S2A7.690	80	16	3.56 ns	42.67 **
27	S2A7.880	57	38	11.40 **	3.80 ns
28	S2A7.980	62	33	4.80 *	8.85 **
29	S3A11.1590	78	18	2.00 ns	37.50 **
30	S3A11.450	32	64	3.56 ns	10.67 **
31	S3A11.490	77	19	1.39 ns	35.04 **
32	S3A11.760	38	58	10.89 **	4.17 *
33	S3A11.850	73	23	0.06 ns	26.04 **
34	S3A4.1550	71	25	0.06 ns	22.04 **
35	S3A4.510	27	70	0.42 ns	19.06 **
36	S3A4.530	79	18	2.15 ns	38.36 **
37	S3A4.660	38	58	10.89 **	4.17 *
38	S3A4.890	74	23	0.09 ns	26.81 **

Table 4.13 contd.

S.No.	Marker Loci	Presence (1s)	Absence (0s)	Chi Square values (Expected ratio 3:1 or 1:3)	Chi Square values (Expected ratio 1:1)
39	S3A4.950	64	33	4.21 *	9.91 **
40	S5A8.260	47	50	28.46 **	0.09 ns
41	S5A8.340	61	36	7.59 **	6.44 *
42	S5A8.490	47	49	34.72 **	0.04 ns
43	S5A8.690	48	48	32.00 **	0.00 ns
44	S5A9.110	58	39	11.96 **	3.72 ns
45	S5A9.210	73	24	0.00 ns	24.75 **
46	S5A9.410	54	43	19.33 **	1.25 ns
47	S5A9.510	58	38	10.89 **	4.17 *
48	S5A9.700	61	34	5.90 *	7.67 **
49	PA1.1040	74	23	0.09 ns	26.81 **
50	PA1.220	79	18	2.15 ns	38.36 **
51	PA1.380	63	34	5.23 *	8.67 **
52	PA1.520	70	21	0.18 ns	26.38 **
53	PA1.550	81	16	3.74 ns	43.56 **
54	PA1.590	64	33	4.21 *	9.91 **
55	PA1.805	66	31	2.51 ns	12.63 **
56	PA1.910	68	29	1.24 ns	15.68 **
57	PA1.990	62	35	6.35 *	7.52 **
58	PA8.1050	34	62	5.56 *	8.17 **
59	PA8.130	44	53	21.45 **	0.84 ns
60	PA8.510	56	41	15.43 **	2.32 ns
61	PA8.990	40	57	13.64 **	2.98 ns
62	PA15.1050	49	44	24.69 **	0.27 ns
63	PA15.160	25	70	0.09 ns	21.32 **
64	PA15.165	74	21	0.42 ns	29.57 **
65	PA15.200	63	32	3.82 ns	10.12 **
66	PA15.280	26	69	0.28 ns	19.46 **
67	PA15.290	78	17	2.56 ns	39.17 **
68	PA15.550	33	61	5.12 *	8.34 **

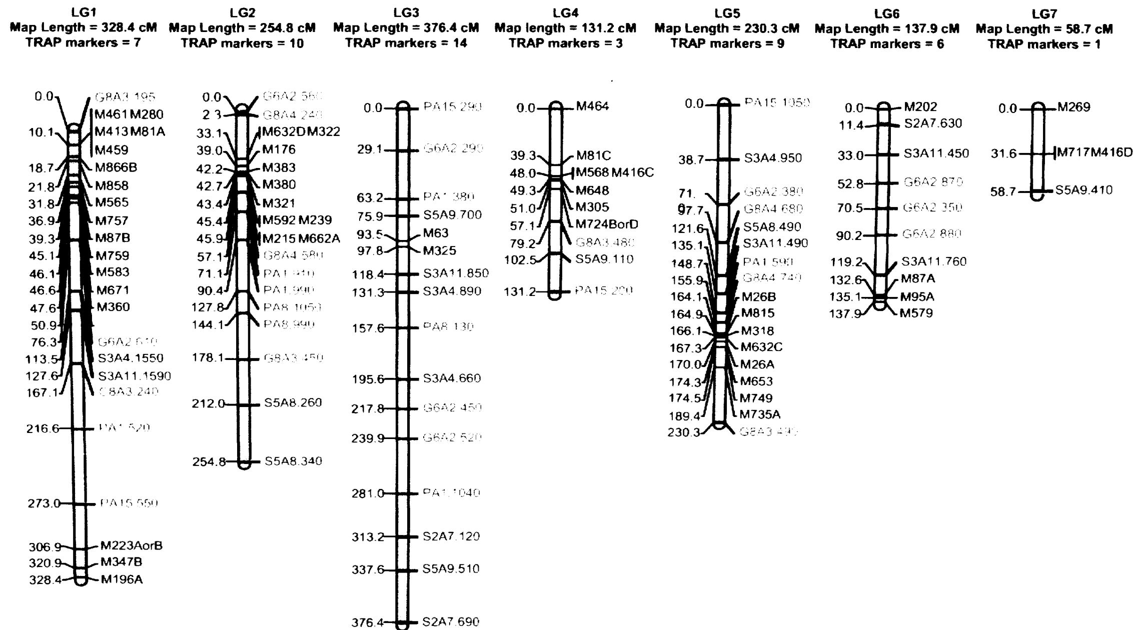
* = significant at 1% level of significance (LOS)
** = significant at 5% LOS
ns = not significant

and PA15.165 and PA15.160, were found co-dominantly segregating in the mapping population. Hence, while constructing the linkage map, the number of TRAP markers was reduced to 64. Of these 64 TRAP markers, 50 were mapped into the framework linkage map along with 54 RFLP markers which were generated previously for the Tift $23D_2B_1$-P1-P5 × WSIL-P8 based pearl millet mapping population (Liu *et al.*, 1994a,b); i.e.,78.1% of TRAP markers detected loci that could be placed on the 7 linkage groups of pearl millet mapping population. The linkage map thus constructed along with the map positions of TRAP markers are given in the Figure 4.19. After adding the new markers, the total map length was 1517.7cM (Kosambi). The newly added TRAP markers were distributed across all seven linkage groups of pearl millet [7, 10, 14, 3, 9, 6 and 1 marker(s) respectively, in LG1, LG2, LG3, LG4, LG5, LG6 and LG7] (Table 4.14).

Linkage group 1 had a total map length of 328.4 cM and was the second longest linkage group for this mapping population. This linkage group consisted of 7 TRAP markers and 18 RFLP markers. This linkage group included 3 TRAP markers designed from GR sequence, 2 from SOD sequence and 2 from P5CS sequence but none of them were very tightly linked to each other.

Linkage group 2, with a total map length of 254.8 cM, contained 10 TRAP markers and 10 RFLP markers and formed the third longest linkage group for this population. Among them, two TRAP markers designed for the GR gene sequence were tightly linked and formed a cluster at the upper end of this linkage group. Linkage group 3 had a total map length of 376.4 cM with 2 RFLP markers and 14 TRAP markers. This is the longest of all the 7 linkage groups and the map distances of the TRAP markers at the lower and upper ends are quite high flanking the two RFLP markers. As this linkage group is usually the shortest, and in this case is anchored by only two RFLP markers, some of the linkages of TRAP markers in this linkage group may be spurious. It will be necessary to assess this on a linkage mapping population having better anchored marker saturation on LG3 before these TRAP markers can confidently be assigned to this linkage group.

Genetic map of a pearl millet population segregating for salt tolerance with distribution of molecular markers across the 7 linkage groups



[Note: The red, blue and green highlighted regions indicate TRAP markers for GR, SOD, and P5CS genes, respectively]

Table 4.14. Linkage group assignment and distribution of markers and length of linkage groups in the Tift $23D_2B_1P1$-P5 × WSIL-P8 based mapping population

Linkage Group	RFLP markers	TRAP markers	Total markers	Length (cM)
LG1	18	7	25	328.4
LG2	10	10	20	254.8
LG3	2	14	16	376.4
LG4	7	3	10	131.2
LG5	8	9	17	230.3
LG6	4	6	10	137.9
LG7	3	1	4	58.7
Total	52	50	102	1517.7

The lengths of linkage groups 4, 5 and 6 were 131.2 cM, 230.3 cM and 137.9 cM, respectively. In linkage group 6, all the 6 TRAP markers (3 for GR gene and 3 for SOD gene) mapped between the RFLP markers M202 and M87A and so appear to provide gap-filling role between these two distantly located RFLP markers. Linkage group 7 had a total length of only 58.7 cM in this cross and was the shortest of all the linkage groups. There were 3 RFLP markers and only 1 TRAP marker in this group. A TRAP marker designed from the SOD sequence was mapped to the lower end of this linkage group.

4.5.5 QTL analysis

4.5.5.1 Phenotypic data analysis

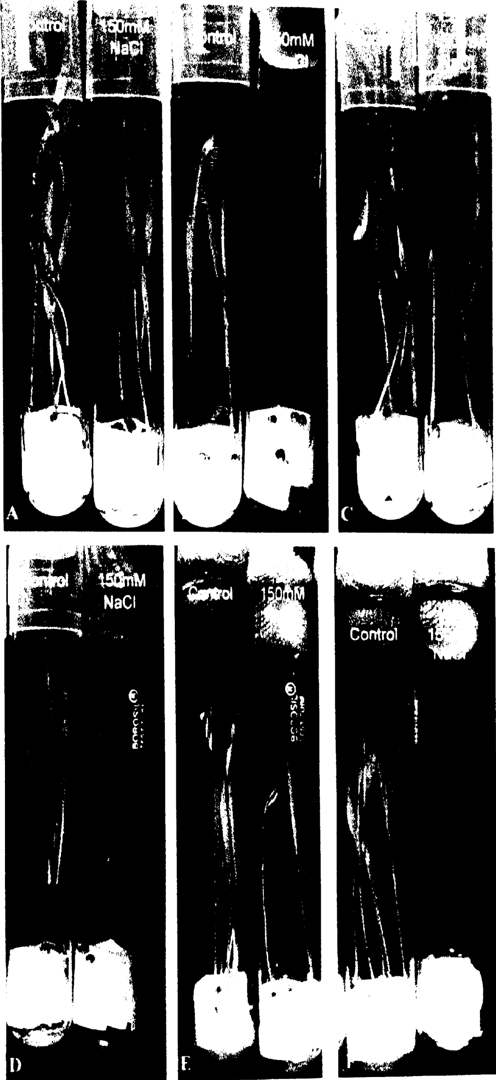
Seeds of the parents and individual progenies of the $F_{2:4}$ mapping population were germinated and grown for 10 days on filter paper boats in balanced nutrient solutions (Hoagland and Arnon, 1938) of pH 6.7, containing two different concentrations of NaCl (0mM and 150mM) (Plate 17). The germination percentage was considerably affected by poor seed quality (due to rain during seed maturation) and dormancy of some progenies included in the mapping population. However this problem was overcome by replicate experiments. The phenotypic data were analyzed for significance of variance using the Residual Maximum Likelihood (ReML) algorithm implemented in the Genstat statistical software package (Genstat, 1995) and the differences in entry means were found significant with respect to all of the observed traits for both environments tested (saline and non-saline medium), as shown in Table 4.15.

4.5.5.2 QTL mapping

Combining the marker data set with the phenotypic traits dataset and the linkage maps permitted evaluation of the ability of the map to detect QTLs for salt stress tolerance. QTL analysis revealed that salt tolerance using germination, shoot and root lengths as measurement has potentially valuable QTLs associated with the TRAP markers.

A total of 9 putative QTLs, viz., 2 for Shoot Length at 150mM NaCl (SL_150), 4 for Relative Shoot Length (Rel_SL), 2 for Root Length at 150mM NaCl (RL_150)

Plate 17. Screening for salinity stress related phenotypic traits for some randomly selected (Tift $23D_2B_1$-P5 × WSIL-P8) $F_{2:4}$ mapping progenies in two salinity levels (0mM and 150mM NaCl)



Note: (A-F) Each pair of test tubes contains an $F_{2:4}$ progeny of the (Tift $23D_2B_1$-P5 × WSIL-P8)-based mapping population].

Table 4.15 contd...

*G.No.	*ENT	*GEcon	Rank	% of mean	*GE150	*GErel	*GEdev	*SLcon	*SL150	*SLrel	*SLdev	*RLcon	*RL150	*RLrel	*RLdev
48	28	41	52	90	22	55.5	9.84	3.8	1.9	49.3	0.95	1.8	1.5	82.8	1.07
49	29	13	92	29	8	71.9	7.54	3.1	0.9	29.0	0.28	2.4	0.4	14.8	-0.37
51	30	86	6	190	0	0.0	-32.01	7.0	0.0	0.0	-2.13	5.9	0.0	0.0	-2.44
53	31	59	25	131	0	0.0	-20.52	8.8	0.0	0.0	-2.82	7.1	0.0	0.0	-3.07
55	32	72	19	158	4	6.7	-21.84	10.6	0.1	0.9	-3.42	8.9	0.0	0.0	-3.94
56	33	55	29	122	0	0.0	-18.82	6.8	0.0	0.0	-2.04	4.4	0.0	0.0	-1.70
62	34	19	84	41	4	23.6	0.70	3.5	0.0	0.0	-0.77	2.0	1.0	50.0	0.46
63	35	28	70	61	10	33.4	2.87	4.1	2.8	67.2	1.74	1.0	0.7	65.0	0.63
64	36	15	89	33	5	16.7	3.19	7.1	1.5	24.2	-0.68	5.6	0.8	13.6	-1.57
69	37	43	43	94	5	8.4	-8.51	8.0	1.0	13.5	-1.50	6.3	0.4	6.4	-2.23
70	38	27	74	59	0	0.0	-6.70	8.1	0.0	0.0	-2.57	4.5	0.0	0.0	-1.76
72	39	41	49	91	10	25.0	-2.87	4.9	0.8	15.6	-0.57	1.6	0.4	26.6	0.10
73	40	54	31	119	5	10.0	-13.40	6.0	0.9	16.4	-0.85	2.0	0.4	20.0	-0.13
74	41	50	36	111	15	30.0	-1.70	6.0	1.2	22.2	-0.52	2.9	0.9	30.0	-0.05
75	42	18	87	40	0	0.0	-3.09	4.1	0.0	0.0	-1.01	1.5	0.0	0.0	-0.27
76	43	78	10	171	10	12.5	-18.39	5.6	3.2	58.1	1.66	1.4	0.3	17.8	0.01
77	44	41	52	90	59	145.8	46.34	4.3	3.2	89.9	2.10	1.5	2.0	130.0	1.68
78	45	68	20	150	63	92.9	38.64	6.7	3.4	50.6	1.36	4.5	1.8	39.7	0.04
79	46	50	36	111	10	20.0	-6.70	7.3	2.0	27.6	-0.24	3.2	0.9	28.1	-0.24
80	47	18	87	40	5	25.0	1.91	3.3	0.0	0.0	-0.67	1.5	0.0	0.0	-0.27
81	48	59	25	131	5	8.4	-15.52	5.1	1.5	29.7	0.11	1.3	1.2	89.6	0.98
82	49	50	36	111	22	45.0	5.80	5.5	2.6	46.7	1.02	5.2	2.8	52.7	0.61
84	50	41	49	91	35	86.3	22.12	9.2	3.2	34.2	0.14	6.7	3.2	46.5	0.24
85	51	23	80	50	22	100.0	17.50	5.1	1.7	32.3	0.25	3.1	2.5	80.1	1.43
86	52	28	70	61	0	0.0	-7.13	1.8	0.0	0.0	-0.09	0.5	0.0	0.0	0.23
87	53	19	84	41	0	0.0	-3.30	4.3	0.0	0.0	-1.06	1.9	0.0	0.0	-0.49
88	54	51	33	113	5	8.4	-12.12	7.5	0.0	0.0	-2.33	2.0	0.4	17.0	-0.17
89	55	82	7	183	64	78.0	32.98	10.2	10.2	106.8	6.85	10.7	8.5	79.2	3.62
92	56	14	91	30	4	23.6	2.82	10.1	4.8	46.5	1.40	6.7	3.3	50.7	0.42
95	57	24	77	52	8	47.1	2.57	6.8	0.8	10.7	-1.29	2.3	0.3	10.0	-0.45
96	58	32	63	70	5	16.7	-3.83	7.4	1.5	20.5	-0.80	8.0	0.5	9.1	-3.00

Table 4.15 contd...

*G.No.	*ENT	*GEcon	Rank	% of mean	*GE150	*GErel	*GEdev	*SLcon	*SL150	*SLrel	*SLdev	*RLcon	*RL150	*RLrel	*RLdev
97	59	64	22	142	36	57.7	13.35	2.3	3.8	50.0	3.46	7.1	7.0	98.6	3.94
99	60	46	40	102	13	30.3	-1.50	6.7	4.4	65.5	2.35	4.0	1.5	37.5	-0.01
100	61	42	45	92	0	0.0	-13.08	8.9	0.0	0.0	-2.86	5.3	0.0	0.0	-2.16
101	62	23	80	50	8	34.0	3.50	2.1	0.4	20.0	0.19	0.6	0.3	60.0	0.45
102	63	19	84	41	13	72.1	9.70	5.0	1.0	19.2	-0.35	2.5	1.3	50.0	0.48
103	64	55	29	122	22	41.7	3.68	7.0	4.0	57.0	1.86	4.0	3.5	88.9	1.99
106	65	32	60	72	13	46.5	4.24	6.5	2.6	39.6	0.63	2.5	0.7	26.0	-0.12
107	66	14	90	31	9	75.0	7.61	7.3	1.0	13.6	-1.23	3.5	1.0	28.6	-0.26
108	67	46	40	102	10	20.0	-5.00	4.5	1.5	33.4	0.34	3.1	0.8	23.1	-0.33
110	68	28	70	61	15	53.4	7.87	6.0	2.1	36.6	0.41	4.5	2.4	52.2	0.59
111	69	9	94	20	0	0.0	0.74	3.7	0.0	0.0	-0.83	1.3	0.0	0.0	-0.15
114	70	36	55	81	0	0.0	-10.96	5.2	0.0	0.0	-1.42	1.9	0.0	0.0	-0.47
115	71	19	84	41	0	0.0	-3.30	6.9	0.0	0.0	-2.08	6.7	0.0	0.0	-2.88
116	72	9	94	20	0	0.0	0.74	4.1	0.0	0.0	-1.01	1.5	0.0	0.0	-0.27
117	73	36	55	81	3	10.6	-7.46	5.0	2.0	44.5	0.64	1.2	0.8	83.3	0.63
118	74	72	17	160	49	67.2	22.73	8.6	3.6	41.2	0.81	3.0	5.9	195.0	4.84
119	75	46	40	102	10	20.0	-5.00	6.8	0.8	10.7	-1.29	2.6	1.5	60.0	0.67
120	76	32	60	72	0	0.0	-9.26	4.8	0.0	0.0	-1.26	2.5	0.0	0.0	-0.77
122	77	46	40	102	10	21.9	-5.00	9.1	0.3	2.8	-2.71	6.1	1.5	24.6	-1.07
123	78	28	70	61	0	0.0	-7.13	7.0	0.0	0.0	-2.12	5.5	1.0	18.2	-1.26
124	79	24	77	52	10	33.4	4.57	6.8	2.2	32.1	0.21	1.6	1.0	66.7	0.66
125	80	41	49	91	5	11.9	-7.87	10.7	1.5	14.8	-2.06	9.3	0.4	4.2	-3.77
127	81	29	66	63	15	37.5	7.44	8.4	3.0	33.4	0.32	2.0	1.8	97.2	1.22
130	82	32	63	70	17	54.6	8.67	5.5	4.2	77.8	2.72	3.5	2.8	78.5	1.49
131	83	23	80	50	0	0.0	-5.00	6.1	0.0	0.0	-1.79	2.5	0.0	0.0	-0.77
132	84	64	22	142	31	50.5	8.35	7.1	4.4	61.3	2.17	1.7	1.8	104.2	1.35
133	85	78	10	171	36	47.0	8.11	9.8	4.9	50.2	1.67	4.0	2.8	72.2	1.24
135	86	41	49	91	0	0.0	-12.87	7.9	0.0	0.0	-2.48	1.8	0.0	0.0	-0.43
136	87	32	60	72	5	20.0	-4.26	8.1	0.0	0.0	-2.54	4.6	1.8	38.9	-0.08
137	88	74	14	163	0	0.0	-26.70	10.3	0.0	0.0	-3.42	3.7	0.0	0.0	-1.39
138	89	96	3	212	22	23.6	-13.76	12.4	5.9	47.5	1.64	7.7	5.7	73.4	2.24

Table 4.15 contd...

*G.No.	*ENT	*GEcon	Rank	% of mean	*GE150	*GErel	*GEdev	*SLcon	*SL150	*SLrel	*SLdev	*RLcon	*RL150	*RLrel	*RLdev
139	90	36	55	81	5	12.5	-5.96	7.3	0.0	0.0	-2.24	3.7	0.8	18.7	-0.64
140	91	27	74	59	0	0.0	-6.70	6.8	0.0	0.0	-2.04	3.0	0.0	0.0	-1.02
145	92	100	1	221	5	5.0	-32.97	6.9	0.0	0.0	-2.09	5.7	0.6	10.0	-1.78
147	93	78	10	171	22	29.2	-5.89	9.2	3.8	40.3	0.73	8.0	0.9	11.3	-2.63
148	94	51	33	113	14	26.2	-3.12	6.5	1.9	28.9	-0.06	4.7	1.3	26.1	-0.64
151	95	74	13	164	5	5.6	-21.91	7.8	0.0	0.0	-2.43	6.5	0.4	6.1	-2.36
153	96	58	27	129	46	78.5	25.69	11.2	7.9	70.0	4.08	9.1	7.0	76.8	2.95
159	97	64	22	142	10	15.8	-12.65	8.7	4.8	55.0	1.98	4.0	2.7	67.8	1.14
SE (±)		5.05			6.57	16.04	6.67	0.59	0.85	14.01	0.88	0.42	0.57	22.54	0.64
Mean		45.19			14.65	29.23	0	6.54	1.99	29.01	0.03	3.89	1.46	37.19	0.01
CV (%)		15.82			63.37	77.62	-9E+06	12.76	60.27	68.29	4063.59	15.17	55.46	85.73	14805
FRatio		22.74			7.7	3.48	5.11	14.19	7.32	4.53	5.76	35.54	14.75	2.98	8.04
$h^{2,1}$		0.92			0.77	0.55	0.67	0.87	0.76	0.64	0.7	0.95	0.87	0.5	0.78
$h^{2,2}$		0.96			0.87	0.71	0.8	0.93	0.86	0.78	0.83	0.97	0.93	0.66	0.88

$h^{2,1}$ = operational heritablity calculated on the basis of entry mean values
$h^{2,2}$ = operational heritablity calculated on the basis of plot values

* [Note: G.No = Genotype Number; ENT = Entry number; GEcon= germination at control (0mM NaCl); GE150= germination at 150mM NaCl; GErel= relative germination (%) compared to non-saline control; GEdev = deviation in germination from non-saline control; SLcon= shoot length at control (0mM NaCl); SL150= shoot length at 150mM NaCl; SLrel = relative shoot length (%) compared to control; SLdev= deviation in shoot length compared to control; RLcon= root length at control (0mM NaCl); RL150= root length at 150mM NaCl; RLrel = relative root length (%) compared to control; RLdev= deviation in root length compared to control]

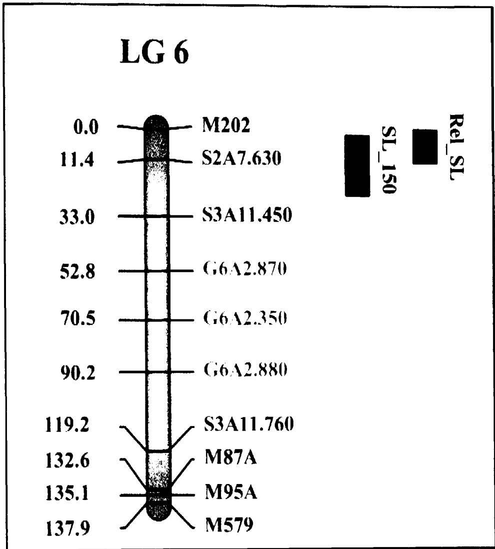
and 1 for Relative Root Length (Rel_RL) were identified by SIM (Simple Interval Mapping) using MapMaker/QTL version 1.1b (Lincoln *et al.*, 1992b) at a LOD threshold value of 3.0 and CIM (Composite Interval Mapping) using PlabQTL version 1.2 (Utz and Melchinger, 2003) (Table 4.16). None of the putative QTLs detected were common across the traits and stress treatments. No QTLs were detected for germination (%) of pearl millet under salt stress. Preliminary QTL analysis by CIM using PlabQTL version 1.2 (Utz and Melchinger, 2003), revealed that Shoot Length in 150mM NaCl (SL_150) was associated with a major QTL mapping between RFLP marker locus M202 and TRAP marker S2A7.630 at the top of LG6 (Table 4.16 and Fig. 4.20), which explained approximately 16% of the adjusted phenotypic variance for this trait among the mapping population progenies. Similarly, a significant QTL for Relative Shoot Length (Rel_SL) was detected in this same marker interval, and explained approximately 25% of the adjusted phenotypic variation in the ability to maintain shoot length under saline conditions at levels comparable to control conditions (Table 4.16 and Fig. 4.20). This QTL was seen to increase shoot length by 18% and was over-dominantly inherited from the salt-tolerant parent.

Other putatively identified QTLs governing root length and shoot length under saline conditions (Table 4.16) were unreliable due to large gaps in the linkage map, probably due to the fact that the TRAP markers were generated for the $F_{2:4}$ generation of the mapping population (while the RFLP markers had been generated on the F_2 generation itself). Hence, these QTLs need to be confirmed using a proper F_2 or RIL mapping population and related phenotypic traits in later studies.

Table 4.16 Details of the putative QTLs identified associated with salinity tolerance traits using $F_{2:4}$ self bulk progenies of the pearl millet (Tift $23D_2B_1$—P1-P5 × WSIL-P8)-based mapping population and CIM implemented in PlabQTL

S.No	Trait	QTL	LG	Left Marker	Marker Interval	Support Interval (cM)	Peak LOD	R^2%	Effect (add)	Effect (dom)	(d*4)/a Ratio	Inheritance
1	**SL_150**	1	3	PA15.290	46-47	0-12	2.72	12.6	1.002	-0.337	-1.3453	Increased SL_150 is recessively inherited from salt-sensitive parent.
		2	**6**	**S2A7.630**	**90-91**	**2-24**	**3.52**	**15.6**	**-0.693**	**-1.044**	**6.0260**	**Increased SL_150 over-dominantly inherited from salt-tolerant parent.**
							LOD = 4.95	**Adj. $R^2\%^2$ = 15.8**				
2	**Rel_SL**	1	2	G6A2.560	26-27	0-24	3.15	15.0	-14.803	-0.207	0.0559	Increased Rel_SL is additively inherited from salt-tolerant parent
		2	4	M648	66-67	48-52	2.79	13.9	-26.679	2.376	-0.3562	Increased Rel_SL is additively inherited from salt-tolerant parent
		3	5	PA1.590	78-79	134-156	3.50	16.2	-14.877	-3.410	0.9169	Increased Rel_SL is dominantly inherited from salt-tolerant parent
		4	**6**	**M202**	**89-90**	**0-12**	**5.18**	**24.7**	**-18.679**	**-13.29**	**2.8460**	**Increased Rel_SL is over-dominantly inherited from salt-tolerant parent**
							LOD = 10.08	**Adj. $R^2\%^2$ = 26.8**				
3	**RL_150**	1	3	PA8.130	54-55	156-196	3.80	17.5	-4.179	-6.849	6.5556	Increased RL_150 over-dominantly inherited from salt-tolerant parent
		2	4	M724BorD	68-69	52-66	3.13	15.4	1.555	-0.208	-0.5350	Decreased RL_150 is partially dominantly inherited from salt-tolerant parent
							LOD = 4.29	**Adj. R^2% = 6.8**				
4	**Rel_RL**	1	7	M269	99-100	0-32	2.69	13.6	18.676	10.61	2.2724	Increased Rel_RL is over-dominantly inherited from salt-sensitive parent
							LOD = 3.96	**Adj. R^2% = 11.7**				

Figure 4.20. Promising QTLs for shoot length and relative shoot length under saline conditions identified on LG6 of pearl millet



[Note: SL_150= Shoot Length at 150mM NaCl, Rel_SL= Relative Shoot Length at 150mM NaCl compared to the non-saline control]

DISCUSSION

CHAPTER 5
DISCUSSION

The problem of salinity is increasing worldwide and therefore efforts are being made to combat it. One of the strategies to deal with salinity is to develop more salt tolerant crops and this has increased the need to understand the mechanisms of salt tolerance. Pearl millet adapts well to diverse environments and drought, and is easy to cultivate. Pearl millet grain has high nutritive value for food and feed rations. It is therefore, a promising cereal crop for the arid and semi-arid regions. In fact, pearl millet is the only cereal that can be grown in some of the hottest and driest regions where rainfed agriculture is possible in India and Africa and so plays a critical role in food security in such areas. It is the source of food, feed and fuel for the poorest of the poor people living in these marginal lands of the arid and semi-arid tropics. However, such areas suffer from salinity problems too. Compared to many other cereals, pearl millet has received relatively little research attention. The development of pearl millet varieties or hybrids with improved salt tolerance could benefit the people of semi-arid and arid regions, improving their livelihoods. Genetic variation in the effects of salinity on germination, growth and yield of pearl millet, thus, requires critical investigation.

Molecular-marker based breeding has tremendous potential to identify the genes or genomic regions responsible for a particular trait of interest. With the availability of large numbers of DNA sequences in public databases with putative functions, the task of bridging this information with a particular phenotype can easily be accomplished using molecular markers. The approach of this study was to focus on the responses of a set of genetically diverse pearl millet inbred lines to salinity in order to assess differences in their salt tolerance and to develop molecular markers associated with salinity tolerance in this crop.

5.1 Screening and selection of pearl millet germplasm for salinity tolerance

Salt tolerance is considered to be a developmentally regulated phenomenon, with germination and early seedling growth stages usually being the most sensitive (Maas *et al.*, 1983). Tolerance at one stage of plant development does not necessarily correlate with the tolerance at other developmental stages (Shannon, 1985). However, salt tolerance at the germination and seedling stage examined in solution culture persisted through to the mature plant in sorghum (Azhar and McNeilly, 1987), barley (Martinez-Cob *et al.*, 1987), maize (Ashraf and McNeilly, 1989; Maiti *et al.*, 1996), alfalfa (Al-Khatib *et al.*, 1994) and millet (Kebebew and McNeilly, 1995). Almost all work on salt tolerance in different crop species reported previously (Kingsbury and Epstein, 1984; Norlyn and Epstein, 1984, Allen *et al.*, 1985, Sayed, 1985, Singh and Rana, 1989, Cramer *et al.*, 1991) has included plant assessment at seedling growth stages. Assessment in solution culture during germination and seedling growth stages can provide a rapid, accurate and less expensive method of initial screening of a large number of accessions for salt tolerance (Singh and Chatrath, 2001). Differences in relative NaCl tolerances of ten seedlings based upon root length data clearly showed that such variation exists between and within maize accessions examined by Khan *et al.* (2003).

Effects of salinity are seen to vary during plant development, thus giving way to arguments concerning methods about selection criteria for distinguishing tolerant and sensitive plants. Germination percentage, shoot and root lengths, seedling survival are generally used as simple measurements of selection criteria and have been successful in sorghum (Azhar and McNeilly, 1989), maize (Rao, 1997), rice (Yeo *et al.*, 1990) and wheat (Prakash and Sastry, 1992; Noori and McNeilly, 2000). In the present study, these same parameters were used for screening a small set of genetically diverse pearl millet inbreds. The effects of varying concentrations of NaCl on shoot length, fresh and dry weights,as well as shoot proline content and accumulation of K^+ and Na^+,were reported earlier in maize (Çiçek and Çakirlar, 2002), wheat (Almansouri *et al.*, 2001), rice (Lee *et al.*, 2003) and barley (Bagci *et al.*, 2003). Boubaker (1996) showed that

germination and seedling characteristics are also viable criteria for selecting salt tolerance in *durum* wheat. Hence, to assess exploitable genetic variability in pearl millet for salinity tolerance and to detect the salt-sensitive and salt-tolerant lines among a set of 28 pearl millet inbreds, these lines were evaluated *in vitro* for germination and early seedling growth characters under non-saline control conditions and three levels of NaCl.

5.1.1 Morphological parameters

5.1.1.1 Effect of salt stress on germination

Analysis of variance (ANOVA) (Table 4.1) among the 28 pearl millet inbreds revealed significant genotypic differences for germination percentage. Significant salinity level × genotype interaction effects ($P<0.05$) were also observed for germination, indicating that the differences between genotypes depended on the salinity level (and thus that the inbreds differed in their relative salinity tolerance during germination) as was also observed for barley genotypes in a similar study (Othman *et al.*, 2006). Germination percentage of the pearl millet inbreds was strongly affected by salinity levels and increased salt concentration caused a decrease in final germination percentage. These results were in agreement with Basalah [1991], who found that high levels of soil salinity can significantly inhibit seed germination. In general, both halophytes (Ungar, 1996) and glycophytes (Varshney and Baijal, 1977) respond in a similar manner to increased salinity stress during germination; with both a reduction in the percentage of seeds germinating and a delay in the initiation of the germination process. Inhibition of seed germination in glycophytes under salt stress could be attributed to osmotic stress or to specific ion toxicity [Huang and Redmann, 1995]. Moderate stress intensities seemed to delay germination while higher stress intensities significantly inhibited the final germination percentages of pearl millet similar to the results reported by Almansouri *et al.* (2001) in wheat.

Even though salt tolerance during germination differs from that at later stages of plant development (Ashraf *et al.,* 1997; Mano and Takeda, 1997a), good germination under saline conditions is essential because it is the first stage of

plant growth. From this perspective, it is clear that five pearl millet lines (Tift $23D_2B_1$-P1-P5, P301-17Bk, 841B-P3, ICMB 95333 and ICML22) with high germination percentages at higher salt level (150mM NaCl), have advantages over the other genotypes that lost their ability to germinate at higher salt concentrations. However, most pearl millet inbreds considered were found to possess a moderate level of tolerance to salinity (i.e. ability to germinate up to 75mM NaCl concentration). But, germination of seeds of halophytes is inhibited or severely reduced only at or above 250mM NaCl (Malcolm *et al.*, 2003). Even barley, which is considered one of the most salt-tolerant cereals, does not survive at salt concentrations higher than 250mM NaCl (Munns *et al.*, 2002). Some of the grain such as sorghum, maize, barley, rice, cowpea and wheat (and legumes such as cowpea) are relatively tolerant to salinity during germination, but sensitive to salinity at the seedling and early vegetative growth stages, and then again become tolerant as they approach maturity (Akbar and Yabuno, 1977; Ashraf, 1994). In contrast, *durum* wheat is less tolerant to salinity at germination than after third-leaf stage (Francois *et al.*, 1986). Selection for salt tolerance can also be undertaken where the plant is only sensitive at one particular growth stage, as in sugar-beet (Bernstein and Hayward, 1958).

5.1.1.2 Effect of salt stress on length, fresh and dry weights of shoots and roots

Our results show that lines of pearl millet tolerant, moderately tolerant and sensitive to salinity differed significantly in all of the observed parameters, including reduction in shoot lengths, fresh weights and dry weights of shoots and roots in response to salinity stress (Fig. 4.2 to 4.7). Relative shoot and root length reduction were the traits most affected by salinity stress in all lines followed by shoot dry weight and then fresh weight. Similarly, sorghum when exposed to high levels of NaCl exhibited reduced shoot growth (Bernstein *et al.*, 1993; deLacerda *et al.*, 2003). Reduction in shoot and root dry weights could perhaps be used as one of the good parameters to characterize salinity tolerance in pearl millet at the seedling stage as opined by Lee *et al.* (2003) for rice. The shoot/root ratios based on lengths of 10-day old pearl millet seedlings decreased with increased salinity levels. In rice varieties, a positive correlation between the shoot/root ratio and the tolerance to NaCl was found (Lutts *et al.*,

1996b). According to Shannon *et al.* (1994), under field conditions, the decrease in the shoot/root ratio under saline conditions, allows a better use of the soil moisture and nutrients. Plant roots are the first organs to become exposed to salinity after germination, and root growth is particularly sensitive to salinity (Cramer *et al.*, 1988). In many cases, pearl millet seedling root lengths were observed to increase with increased salt concentration [Table 4.3]. Root length is an important indication of salt stress tolerance in plants because roots provide contact with the soil and absorb water and nutrients from it. For this reason, root length provides an important clue for the salt stress response.

In the present study, the relative reduction of shoot length and shoot dry matter (shoot dry weight, mg/seedling) at various salinity levels was less in the most tolerant lines than in the sensitive lines (Tables 4.6 and 4.7). Salinity tolerance is measured as relative value, this value being more important in assessing plant species of diverse origin (Shannon, 1984; Ashraf and Waheed, 1990). Twenty-nine accessions of *durum* wheat assessed for their ability to sustain growth under saline conditions at the seedling stage, which were measured as absolute and relative values (Noori and McNeilly, 2000), exhibited variation similar to that observed in the present studies.

A general observation from the screening results is that there was grouping of traits under study during germination and early seedling growth in the genotypes that match with the categories made (sensitive, moderately tolerant and highly tolerant to salt stress) (Table 4.8). These results varied from that of rice (Yeo *et al.*, 1990), where although there was useful varietal variation for each of the characters investigated, the desirable characters were scattered amongst different genotypes and hence, there was no grouping of these salinity tolerance traits in a single genotype.

5.1.2 Biochemical parameters

Understanding the mechanisms of salt stress tolerance is expected to lead to more effective means to breed or genetically engineer salt tolerant crops. Salt tolerance research also represents an important part of basic plant biology, contributing to our understanding of subjects ranging from gene regulation and

signal transduction to ion transport, osmoregulation and mineral nutrition as reviewed by various workers (Munns *et al.*, 2006; Ma *et al.*, 2006; Sairam and Tyagi, 2004; Borsani *et al.*, 2003; Munns, 2002; Zhu, 2001a,b, 2002; Grover *et al.*, 2001; Yeo, 1998; Smirnoff, 1998; Neumann, 1997; Gorham *et al.*, 1985). Hence, a study of these aspects may help understand the possible mechanisms for salt tolerance in pearl millet. Furthermore, if the mechanisms of salt tolerance at the germination and early seedling stages can be defined, they will be extremely valuable in breeding programs that are aimed at improving crop plant resistance to salinity. Many plants, such as extreme halophytes, display Na^+ dependence for optimal growth and development and develop specialized structures such as salt glands and bladders to accommodate high salt concentrations in tissues (Glenn *et al.*, 1999). Other plants develop whole plant strategies for avoiding stress such as accelerated completion of ontogeny. However, these specialized adaptations are lacking in most major crop species. Further, the precise impact of osmotic and ionic effects on cell growth, division, phytohormone balance, and death in the context of the whole plant are complex and require further investigations (Munns, 2001). Therefore, emphasis is placed on the molecular genetic mechanisms controlling osmotic regulation at the cellular level, mainly because the action and regulation of most osmoregulatory components was not fully explored in the context of the whole plant. The mechanisms examined to investigate salt tolerance in our studies include accumulation of proline and ions, and activities of antioxidative enzymes.

5.1.2.1 Proline accumulation

One metabolic response to salt stress in a majority of plants is the synthesis of compatible osmolytes. They not only act as osmotic balancing agents but also protect sub-cellular structures and reduce oxidative damage caused by free radicals that are produced as a result of exposure to high salinity levels (Hong *et al.*, 1992; Hare *et al.*, 1998; Kavi Kishor *et al.*, 2005). Osmolytes include sugars, polyols, amino acids like proline and tertiary and quaternary ammonium, and sulphonium compounds such as glycine betaine (Rhodes and Hanson, 1993). The transient accumulation of certain metabolites, such as proline, might serve as a safety valve to adjust cellular redox state during stress

(Shen *et al.*, 1999; Kuznetsov and Shevyakova, 1999; Kavi Kishor *et al.*, 2005). Some Australian halophytic *Melanleuca* species are salt tolerant, and this has been attributed to their ability to accumulate large quantities of osmoprotectants such as proline and proline betaine (Naidu 2003). Whole plant free proline levels were observed to increase with increasing salinity levels in all of the pearl millet lines included in this study, irrespective of their tolerance to salt. Significantly greater proline accumulation was observed in the tolerant lines with increasing salinity levels as compared to the sensitive lines. Perhaps this enables the tolerant genotypes to cope with the salt stress conditions more efficiently. Similar increases in proline content was also reported in rice (Kavi Kishor, 1988; 1989; Dubey and Rani, 1989), pigeonpea (Rao and Rao, 1981), niger (Sarvesh *et al.*, 1996) and many other plants (Delauney and Verma 1993) in response to increasing salinity levels. Proline accumulation was also significant under salinity stress in salt tolerant cultivars of green gram (Misra *et al.*, 2006), and is correlated with salt tolerance of many higher plants (Bray *et al.*, 1991; Perez-Alfocea and Larher, 1995; Lopez *et al.*, 1994; Sarvesh *et al.*, 1996; Ghoulam *et al.*, 2002; Girija *et al.*, 2002). Higher proline accumulation is related to salt tolerance in the salt-tolerant genotypes, and does not occur as a consequence of tissue dehydration or tissue reaction to stress damage (Misra and Gupta, 2005). Accumulation of free proline is correlated with tissue Na^+ concentration for numerous plant species. This strongly suggests a possible role of proline in osmoregulation during salt stress (Goas *et al.*, 1982; Levitt 1980). Proline is an important osmoprotectant in plants and can protect the photosynthetic machinery against salt induced damage (Sivakumar *et al.*, 2000). In rice roots exposed to salt stress, more accumulation of proline was correlated with increasing concentrations of NaCl (Khan *et al.*, 2002) as is also noticed in the present study. Proline was shown to protect PEG-induced precipitation of some enzymes and protein complex *in vitro* (Paleg *et al.*, 1985). Proline may act as an enzyme-stabilizing agent in salt-stressed tissues (Demir and Kocacaliskan, 2001) or it may stabilize sub-cellular structures and scavenge free radicals (Hare *et al.*, 1996). Proline was also shown *in vitro* to reduce enzyme denaturations caused due to high temperature, NaCl stress, etc. (Hamilton and Heckathorn, 2001). NaCl curtailed carboxylase activity of

Rubisco and enhanced the oxygenase activity as was shown by Sivakumar *et al.* (2000). But salt-stress induced oxygenase activity was suppressed by proline even at a concentration of 50mM NaCl. The above findings support the view that proline plays a critical role in protecting photosynthetic activity under stress. Additionally, proline acts as a reserve source of carbon, nitrogen and energy during recovery from stress (Alia *et al.*, 1991; Zhang *et al.*, 1997). Among various compatible solutes, proline is the only molecule that was shown to protect plants against singlet oxygen and free radical induced damages (Alia *et al.*, 1997). Since proline can act as a singlet oxygen quencher (Alia and Pardhasaradhi, 1993), and as a scavenger of OH· radicals, it is able to stabilize proteins, DNA and membranes (Hamilton and Heckathorn, 2001; Sivakumar *et al.*, 2000; Alia *et al.*, 1991; Bellinger and Larher, 1987; Fahrendorf *et al.*, 1995; Alia *et al.*, 1997; Floyd and Zs-Nagy, 1984; Paleg *et al.*, 1984; Rudolph *et al.*, 1986; Anjum *et al.*, 2000; Smirnoff and Cumbes, 1989; Matysik *et al.*, 2002; Kavi Kishor *et al.*, 2005). Hydroxy-radical scavenging activity has been measured for sorbitol, mannitol, myo-inositol and proline and it was found that proline is an effective hydroxy radical scavenger (Alia *et al.*, 1997; Smirnoff and Cumbes, 1989). Thus, proline is not only an important molecule in redox signaling, but also an effective quencher of reactive oxygen species formed under salt, metal and dehydration stress conditions in all plants, including algae (Alia and Pardhasaradhi, 1991; Kavi Kishor *et al.*, 2005).

5.1.2.2 Accumulation of Na^+, K^+ and other ions under salt stress

Plants depend upon the maintenance of low cytoplasmic Na^+ and Cl concentrations and a high K^+/Na^+ ratio under salt stress, because K^+ counteracts the inhibitory effects of Na^+ and Li^+. Most plant cells maintain cytosolic K^+ concentrations in the range of 100–200 mM and Na^+ values in the low mM range (1– 10 mM) up to a maximum of 100 mM (Maathuis and Amtmann, 1999). In contrast to K^+, Na^+ is not essential for, but facilitates volume regulation and growth in most plants. However, at high concentrations, Na^+ limits growth (Blumwald, 2000). In the present studies, sensitive lines showed more accumulation of Na^+ when compared to K^+. All the tolerant lines recorded K^+/Na^+ ratio >1 indicating higher accumulation of K^+ than Na^+. Less

accumulation of Na^+ and more accumulation of K^+ are also regularly observed in salt-tolerant varieties of rice (Vaidyanathan *et al.*, 2003; Sangam, 1995). Lower uptake of Na^+ and comparatively higher accumulation of K^+ seems to be one of the mechanisms of their tolerance to salinity stress especially in the tolerant lines. Optimal accumulation of K^+ facilitates ion homeostasis which is otherwise disturbed under excess Na^+ concentrations. Bread wheat (*Triticum aestivum*) restricts Na^+ transport to leaf tissues through Na^+ exclusion and maintains high selectivity of K^+ over Na^+ (Gorham *et al.*, 1986; Gorham, 1993). The high K^+:Na^+ ratio in leaves of *T. aestivum* and *T. tauschii* is partially attributed to the enhanced K^+/Na^+ discrimination character (Gorham *et al.*, 1990; Gorham, 1993; Dvora˙k *et al.*, 1994). Similarly, high K^+:Na^+ ratios in leaves of the wild *Hordeum* species indicate mechanisms for Na^+ exclusion and K^+/Na^+ selectivity in these species. However, wild *Hordeum* species with the exception of *H. murinum* appear to maintain K^+/Na^+ selectivity even at high salinity levels (*i.e.* 450 mM NaCl) (Garthwaite *et al.*, 2005). By contrast, the K^+/Na^+ discrimination trait in *T. aestivum* and *T. tauschii* is most apparent at relatively low salinity levels (50 mM) (Gorham, 1993; Gorham *et al.*, 1997). The maintenance of a high cytosolic K^+/Na^+ ratio is significant for plant growth during salt stress (Glenn *et al.*, 1999). The yeast halotolerance gene (HAL1) facilitates K^+/Na^+ selectivity and salt tolerance of cells. Over expression of HAL1 gene in *A. thaliana* resulted in less Na^+ accumulation and promoted salt tolerance (Yang *et al.*, 2001). Similarly, Rus *et al.* (2001) found that a high affinity K^+ transporter (AtHKT1) from *A. thaliana* functions as a selective Na^+ transporter and also mediates K^+ transport.

To avoid cellular damage and nutrient deficiency, plant cells need to maintain adequate K^+ nutrition and a favourable K^+/Na^+ ratio in the cytosol (Niu *et al.*, 1995; Serrano *et al.*, 1999). Excessive concentrations of Na^+ ions at the root surface may disrupt plant K^+ acquisition and nutrition that is vital for the maintenance of cell turgor, membrane potential, and the activities of many enzymes (Lazof and Bernstein, 1999). Once Na^+ enters into the cytoplasm, it has a strong inhibitory effect on the activity of many enzymes. This inhibition is also dependent on K^+ levels in the cytoplasm: a high Na^+/K^+ ratio is most damaging. Therefore, Na^+ needs to be compartmentalized into the vacuole away

from the cytosolic enzymes (Flowers *et al.*, 1977). Recent studies identified pathways for Na^+ entry into cells (Zhu, 2000) and vacuoles through vacuolar membrane-bound proteins. Cloning of genes that encode Na^+/H^+ antiporters (Ohta *et al.*, 2002) demonstrated the importance of intracellular Na^+ compartmentation during salt stress. Thus, salt tolerance requires not only exclusion but also sequestration of Na^+ ions. Besides, the acquisition of K^+ is important, whose uptake is severely affected by high external Na^+ concentration due to the chemical similarities of the two ions. Therefore, K^+ transport systems involving good selectivity of K^+ over Na^+ can be considered as an important determinant of salt tolerance in higher plants (Rodriguez-Navarro, 2000).

Tolerant genotypes not only accumulate less Na^+ but also show minimum imbalance in K^+, Ca^{2+} and Mg^{2+} content (Pandey and Srivastava, 1995-97). A similar trend was noticed in pearl millet lines subjected short-term salt stress treatments. The increase in accumulation of Na^+ and Cl^- were comparatively higher in the salt-sensitive pearl millet lines, while increase in accumulation of K^+ and Ca^{2+} were much higher in the moderately tolerant and highly tolerant lines than the susceptible lines. It appears that the increased accumulation of Cl^- in the sensitive lines not only added to the toxic effects in the plant tissue, but also the saline-induced changes in mineral nutrient uptake likely contributed to the reduction of plant growth in these lines, as was observed in *Phaseolus* species by Bayuelo-Jiménez *et al.* (2003). Several salt-tolerant wild *Hordeum* species were shown to possess an exceptional capacity to exclude Na^+ and Cl^- from their shoots (Garthwaite *et al.*, 2005), thus indicating a probable mechanism for lower increase in accumulation of these ions in the tolerant lines of pearl millet than the sensitive lines. Higher increase in Ca^{2+} in the tolerant lines may be due to its involvement as a second messenger in excluding Na^+ out of the cells via the SOS (salt overlay sensitive) pathway (Zhu, 2003).

5.2 Antioxidative enzyme activity

Among the secondary stresses imposed by high salinity, oxidative stress is an important constraint for salt tolerance. Many studies have implied that salt stress could generate reactive oxygen species (ROS) in plants (e.g. Burdon *et al.*, 1996; Shen *et al.*, 1997; Tsugane *et al.*, 1999; Hong *et al.*, 2000). ROS include

singlet oxygen, hydrogen peroxide, hydroxyl radicals, and superoxide anions. ROS have damaging effects on cellular structures and macromolecules such lipids, enzymes and DNA. Detoxification of these compounds by effective enzymatic machinery contributes to alleviation of salt stress.

It has been demonstrated that salinity-induced oxidative stress and lipid peroxidation is frequently used as an indicator of oxidative stress when plants are subjected to salinity [e.g. in *Morus alba* (Sudhakar *et al.*, 2001), *Lycopersicon esculentum* (Mittova *et al.*, 2002), *Beta vulgaris* (Bor *et al.*, 2003), *Oryza sativa* (Vaidyanathan *et al.*, 2003), and *Gossypium hirsutum* (Meloni *et al.*, 2003)]. To overcome the oxidative stress, plants make use of complex antioxidative machinery. Relatively higher activities of ROS-scavenging enzymes have been reported in stress-tolerant genotypes when compared to stress-sensitive ones, suggeting that the antioxidative system plays an important role in plant tolerance against environmental stresses. Salt-tolerant genotypes generally can protect themselves from salt-induced oxidative stress by maintaining higher concentrations of osmolytes, antioxidative molecules such as anthocyanins and increased antioxidative enzyme activities (Sreenivasulu *et al.*, 2000, Sairam *et al.*, 2002, Bandeoglu *et al.*, 2004, Gossett *et al.*, 1994a) than the salt-sensitive genotypes.

5.2.1 Antioxidative enzyme responses under short-term salt stress

Specific activity of catalase was low in the sensitive lines compared to tolerant ones under salt stress conditions. In the tolerant line, the activity increased by 12-folds by 72h though it declined later on. Such an increase in the activity of CAT was recorded in the tolerant genotypes of *Beta maritime* (halophyte) and the non-halophyte *Beta vulgaris* (Bor *et al.*, 2003) also. Such comparisons were also made between genotypes of rice (Sudhakar. *et al.*, 2001) and wheat (Sairam *et al.*, 2002) differing in salt tolerance. It appears therefore that some genotypes utilize catalase as an effective antioxidative enzyme to convert hydrogen peroxide that is generated during salt stress.

The native activity of SOD appeared lower in the sensitive line when compared to the tolerant lines of pearl millet. This suggests that SOD may function as an

effective ROS scavenger, by converting O^{2-} to H_2O_2 as pointed by Alscher *et al.* (2002). The salt sensitive lines showed a gradual hike in SOD with exposure to salinity till 72h but declined later on. A similar decrease in the level of SOD under salt stress was reported earlier by Santos *et al* (2001) in sunflower. Very high SOD activities were reported under salt stress conditions in several salt-tolerant species compared to the salt-sensitive ones (Sreenivasulu *et al.*, 2000, Gossett *et al.*, 1994a; Acar *et al.*, 2001). Also, the potential role of SOD in the protection against salt stress was examined using transgenic rice plants (Tanaka *et al.*, 1999). Even though a high SOD activity protects the plants against the superoxide radical, it cannot be solely responsible for membrane protection against peroxidation because it converts O^{2-} to H_2O_2, which is also a ROS. This ROS should then be scavenged by other enzymes, such as catalases and peroxidases.

The native activity of GR was lower in salinity sensitive line when compared to the moderately tolerant and tolerant lines. In the sensitive line, the activity of glutathione-S-transferase also declined but slightly increased by 72h. Highest GST activity was observed in the moderately tolerant line while the increase was transient in the tolerant line. Since APX and GR are key enzymes of the ascorbate-glutathione cycle (Noctor and Foyer, 1998), the genes encoding the enzymes in this pathway could be a potential source for generating transgenics that confer salt stress tolerance. In some species salt tolerance was associated with an increase in both APX and GR activities (Bor *et al.*, 2003; Harinasut *et al.*, 2003); only an increase in GR was observed in pearl millet. However, discrepancies were also noticed in several species with regard to the activities of this enzyme (Jogeswar *et al.*, 2006). These could be related to the complexity of this cycle, that have enzymes encoded by multigene families whose products are localized in different cell compartments, and are regulated.differently by stress conditions. Functioning of GSH (reduced glutathione) as an antioxidant compound under salt-induced oxidative stress has received much attention during the last decade. Our results indicated that the content of GSH was less in the susceptible line compared to the tolerant line. Glutathione metabolizes free radicals and also protects the thiol status of proteins (Gilbert *et al.*, 1990). Glutathione and ascorbate mostly exist in the chloroplasts (Meneguzzo *et al.*,

1998; Meneguzzo *et al.*, 1999). They also exist out side the chloroplasts and a decline in the GSH to GSSH ratio usually reflects the predominant oxidation of the cytosolic antioxidant. The increase in total glutathione content in the line 863B-P2 may be increased GR activity in this line and indeed this is reflected in the activity. GSH may be synthesized in the shoots and then transported to roots since roots are the primary organs that suffer salt stress (Ruegsegger *et al.*, 1990). Thus, GSH appears to be an important signal molecule in different defense responses. Oxidative stress produces hydroxyl radicals that are highly toxic to the cells. GSTs conjugate GSH with endogenously produced electrophiles, which results in their detoxification (Williamson and Beverly, 1987). The constitutive as well as salt induced activities of GST were higher in the salt tolerant seedlings under salt stress conditions. This is an indication that salt tolerant species of pearl millet possess effective mechanisms to conjugate with GSH and detoxify the electrophiles when exposed to salt stress conditions. A central nucleophilic cysteine residue may be responsible for the high reductive potential of GSH due to which it scavenges cytotoxic H_2O_2, and reacts non-enzymatically with other ROS as pointed by Larson (1988). The central role of GSH in the antioxidant defense is due to its ability to regenerate another powerful water-soluble antioxidant, ascorbic acid (ascorbate), via the ascorbate-glutathione cycle (Foyer and Halliwell, 1976; Foyer, 1993; Noctor and Foyer, 1998). Glutathione reductase (GR) plays a key role in this cycle during oxidative stress by converting the oxidized glutathione GSSG to GSH and maintaining a high GSH/GSSG ratio (Alscher, 1989; Fadzilla *et al.*, 1997). Higher GSH to GSSG (oxidized form) ratios and GR activities are correlated to acclimation or tolerance in plants subjected to oxidative stress imposed by salt in cotton and *Bruguiera parviflora* (Gossett *et al.*, 1996; Parida *et al.*, 2004a,b). It was previously reported that overexpression of GR in transgenic plants led to elevated levels of GSH, increasing the tolerance to salt and oxidative stresses (Foyer *et al.*, 1991). Roxas *et al.* (2000) reported the alleviation of oxidative stress by over-expression of a tobacco GST in transgenic tobacco seedlings under stress.

While lipid peroxidation was not much in Tift $23D_2B_1$-P1-P5, WSIL-P8 displayed very high levels of MDA under short-term salt stress. An increase in MDA levels

with increasing salt concentration was noticed in the sensitive line ICMB 90111 after 7 days salt treatment, while the moderately tolerant and tolerant lines recorded decrease in MDA levels with increased NaCl concentrations. Such contrasting differences in pearl millet sensitive and tolerant lines indicate that they may possess differential mechanisms to combat the stress. Higher lipid peroxidation in sensitive cultivars of pea (Hernandez *et al.*, 1993) and rice (Dionisio-Sese and Tobita, 1998) were reported earlier; suggesting that the elevated levels of the antioxidative enzymes protect plants against the ROS, thus avoiding lipid peroxidation during salt stress in the tolerant cultivars (Gossett *et al.*, 1994b; Shalata and Tal, 1998; Comba *et al.*, 1998; Shalata *et al.*, 2001). Lipid peroxidation of membranes of higher plants is an indication of free radical-induced oxidative damage under salt stress conditions. This is indicated by MDA levels in the tissues. MDA levels provide an index of oxidative damage due to the inadequate response of the antioxidative systems as evident in several other crop species (Sreenivasulu *et al.*, 2000, Mittova *et al.*, 2002).

5.2.2 Isoenzyme profiles at short term salt stress

Analysis of isoforms of antioxidative enzymes during salinity acclimation will provide important new insights into salt tolerance mechanisms or processes. Superoxide radicals are toxic by-products of oxidative metabolism. Thus, the dismutation of superoxide radicals into H_2O_2 and oxygen by SOD is an important step in protecting the cell. Isoforms of SOD can be divided into three classes based on the metals present in active site: Cu/Zn-SOD, Mn-SOD, and Fe-SOD (Beyer and Fridovich, 1987). Our results indicate the presence of two isoforms of Mn-SOD and one isoform of Cu/Zn-SOD in pearl millet on the basis of activity levels with *in situ* staining technique on the gel. The increase in exposure to salinity stress led to an increased intensity of the Mn-SOD2 and Cu/Zn-SOD isoforms in native gels. Lee *et al.* (2001) observed two isoforms of Mn-SOD and five isoforms of Cu/Zn-SOD in rice, while Fe-SOD isoform was not detected in the activity gels. The difficulty involved in identifying Fe-SOD in different species of higher plants is related to their low enzymatic activity and low expression (Salin and Bridges, 1980; Almansa *et al.*, 1991; Gueta-Dahan *et al.*, 1997). Also, Fe-SOD is usually inactivated by H_2O_2 (Alscher *et al.*, 2002).

Salt-stress increased the activities of leaf mitochondrial Mn-SOD and chloroplastic Cu/Zn-SOD in NaCl-tolerant pea cultivars (Hernandez *et al.*, 1995) and this has been also observed in shoot cultures of rice (Fadzilla *et al.*, 1997).

Our results showed a strong correlation between salt tolerance and the activity of antioxidative enzymes. This suggests that the balance between the activities of H_2O_2-producing and H_2O_2-scavenging enzymes play an important role in providing a defense mechanism against salt-induced oxidative damage in plant cells. Different responses of antioxidative systems to salt stress in different pearl millet lines suggest that there is no universal mechanism incorporating all the antioxidants that lead to ROS detoxification in pearl millet. The genotypes studied here differed significantly in initial antioxidant content, trends of increase/decrease in enzyme activity in response to short-term salinity stress, and lipid peroxidation resulting from salinity stress. The results from this study may provide base-line information and a system necessary to conduct further studies related to the molecular and genetic basis of salinity tolerance in pearl millet to elucidate the importance of the relationship between antioxidant activity and development of salt tolerance.

5.3 Protein profiles

Analysis of the protein profiles of pearl millet on SDS-PAGE revealed that some polypeptides (120kDa) were synthesized in response to salt stress in tolerant lines (P310-17B, 841B-P3, Tift $23D_2B_1$-P1-P5), while synthesis of few existing polypeptides (180kDa and 54kDa) increased under short-term salt stress (in 841B-P3). Similar reports of synthesis of specific proteins under salt stress have been reported in other species also (Misra *et al.*, 2006). Increased synthesis of polypeptides in cells adapted to salinity was also reported (Singh *et al.*, 1985).

Bands of polypetides with molecular weights of about 15kDa, 18kDa, 25kDa, 27kDa and 54kDa were noticed in pearl millet seedlings on exposure to short-term salt stress at 150mM NaCl. The absence of 25kDa protein band in the sensitive lines, while its presence in the moderately tolerant and highly tolerant

lines, indicates its association to salt tolerance. The intensity of 27kDa and 54kDa bands increased in the SDS-PAGE profile when compared to that of the sensitive lines across all the treatments. Such salt stress induced proteins (20-24kDa) were reported earlier in various plants like barley (Ramagopal, 1987; Hurkman and Tanaka, 1987; Hurkman *et al.*, 1991). A protein of 26kDa in *Brassica* (Jain *et al.*, 1993), 15kDa and 26kDa in rice shoots (Shirata and Takagishi, 1990), 26kDa and 27kDa in rice cultured cells (Shirata and Takagishi, 1990), 23kDa in germinating seeds of rice (Rani and Reddy, 1994), 22kDa in *Raphanus sativus* (Lopez *et al.*, 1994), 26kDa in citrus and tomato cells (Ben-Hayyim *et al.*, 1989), 18kDa, 19.5kDa, 21kDa, 26kDa, 34kDa, 35.5kDa, 37kDa and 58 kDa proteins in cultured tobacco cells (Singh *et al.*, 1985) were also recorded earlier. Synthesis of a 26kDa protein was unique in tobacco cells under salt stress. Singh *et al.* (1987a,b) latter named this protein as 'osmotin', because it was synthesized and accumulated by cells undergoing gradual osmotic adjustment to either salt or dessication stress (Chretein *et al.*, 1992; Perez-Alfocea and Larher, 1995). The newly synthesized proteins and also proteins that displayed increased levels of activity under salt stress may have an adaptive role in osmotic adjustments (osmotic as well as ionic components of salt stress) (Shirata and Takagishi, 1990; Singh *et al.*, 1987a,b), thus protecting the key cytoplasmic enzymes and protein synthesizing apparatus against adverse effects of high salt concentrations. The possibility that a variety of environmental stresses may lead to the production of one or more common proteins suggests a general stress tolerance mechanism for abiotic stress and also the possibility that there may be a key set of proteins (Harrington and Alm, 1988) cannot be ruled out from our findings. The polypeptides identified in the present studies were not comparable to those proteins specifically synthesized under heat shock in tobacco cell lines (Harrington and Alm, 1988). Thus, conclusions drawn from the studies of Ben-Hayyim *et al.* (1993) that salt-induced proteins are species-specific and that no similarities exist among them is not consistent with our findings, as a few of the proteins reported in response to salt stress in pearl millet are common (on the basis of molecular weight) with those reported from other sources under salt stress (Singh *et al.*, 1985; Shirata and Takagishi, 1990).

5.4 Effect of GA_3 and $CaCl_2$ on salinity stress

Among the different plant growth regulators, gibberellic acid (GA_3) is capable of reversing the salinity-induced stress on hypocotyl elongation (Banyal and Rai 1983, Smith *et al.,* 1995). Although the effect of gibberellic acid on growth, photosynthesis, enzyme activities and productivity were well studied (Kavi Kishor and Mehta, 1987; Saxena and Pandey, 2001) and its effect on salinity induced inhibition was also found out in various crops (Angrish *et al.*, 2001; Aldesuquy and Ibrahim, 2001), pertinent information with regard to pearl millet was not previously available. Similarly, an important determinant for plant salt tolerance that is relevant to Na^+ and K^+ homeostasis is calcium. It was observed that increased Ca^{2+} supply has a protective effect on plants under NaCl stress. One possible approach to reduce the effect of salinity on plant productivity is the addition of Ca^{2+} to the growth medium. Several earlier reports indicated that supplemental Ca^{2+} (usually up to at least 5mM) may alleviate the reduced growth caused by salinity. A Ca^{2+} signaling pathway regulates a K^+ channel for low potassium response in *Arabidopsis* (Li *et al.,* 2006). Because H_2O_2 production leads to Ca^{2+} changes in plant cells (Pei *et al.,* 2000), it is conceivable then that Ca^{2+} functions as a second messenger in plant response to low-K^+ stress.

The effect of Ca^{2+} and GA_3 in ameliorating salinity stress in two inbred lines of pearl millet, ICMB 90111 (sensitive) and 841B-P3 (tolerant) was investigated in the present study. Because the two lines tested have different degrees of salt tolerance, one of the primary objectives was to determine how these lines responded to the combination of salinity and $CaCl_2$ or GA_3, or salinity and both $CaCl_2$ and GA_3 with respect to seed germination, early seedling growth and proline content. Both $CaCl_2$ and GA_3 were effective in partly ameliorating the adverse effects of salinity stress on germination and early seedl;ing growth measured in terms of length and fresh weights of shoots and roots, as well as on proline accumulation. Likewise, germination percentage growth and grain yield of wheat decreased with increasing salinity levels, but these effects were partially ameliorated by seed treatment with GA_3 (Kumar and Singh, 1996; Ashraf *et al.,* 2002). In another study, wheat seeds after treatment with various

growth regulators, showed the highest percent germination when treated with 20mgL^{-1} GA_3 (Nayyar *et al.*, 1995). Additionally, Huber *et al.*, (1974) found that GA_3 counteracted the effect of NaCl on the *in vivo* activity of carbohydrate metabolism in leaves of *Pennisetum typhoides* (a synonym for *P. glaucum*). Ca^{2+} alleviated the salt-induced inhibition of root elongation more effectively than shoot elongation, especially in the salinity-sensitive pearl millet line ICMB 90111, similar to previous reports in wheat (Kinraide, 1999). The reason for this is that salts in the nutrient solution lower the activity of Ca^{2+} (Cramer and Läuchli, 1986) and root growth is severely affected. Root elongation of maize was not affected by the addition of 80mM NaCl, provided that supplemental Ca^{2+} (10mM) was given. In the absence of exogenous Ca^{2+}, there was a reduction in root growth that was not readily reversed by subsequent supply of Ca^{2+} (Cramer *et al.*, 1988). Similarly, even at 150mM NaCl, elongation of sorghum roots was reduced by only 20% if supplemental Ca^{2+} was given, but by 80% devoid of it (Colmer *et al.*, 1996). The effect of supplemental Ca^{2+} on salt-stressed roots was also related to the ion activities in the external solution (Cramer *et al.*, 1986; Yermiyahu *et al.*, 1997). Application of Ca^{2+} increased the salt-induced proline accumulation especially, in the salt tolerant line of pearl millet as reported earlier in wheat seedlings also (Sadiqov *et al.*, 2002). A similar observation was made with GA_3. Further, a combination of $CaCl_2$ and GA_3 considerably increased the proline accumulation in the salinity-sensitive line ICMB 90111 and increased this almost 6-fold in salinity-tolerant line 841B-P3, as compared to their corresponding salinity-stressed controls. The positive effect of Ca^{2+} may be due to its role in the protection of cell membranes from the adverse effects of salinity (Busch, 1995) or its role in the signal transducing events.

5.5 Development of molecular markers

Of the several marker techniques available till date, SSR markers are currently the best option available to plant breeders for marker-assisted backcrossing programs, because of their ease of use, co-dominant inheritance, high levels of polymorphism, and reasonably even distribution across the nuclear genome. To date, about 100 SSR markers are available for use in pearl millet (e.g., Qi *et al.*,

2001; Allouis *et al.*, 2001; Budak *et al.*, 2003; Qi *et al.*, 2004; Senthilvel *et al.*, 2004). However, a much larger number is required for their widespread application in plant breeding. Therefore, development of additional novel PCR-compatible markers is a valuable objective for the pearl millet research community, especially if these markers can be targeted at genomic regions known to contribute to the control of economically important, genetically complex traits such as salinity tolerance. In the present investigation, the novel TRAP marker approach (Hu and Vick, 2003) was used as this allows PCR-based mapping of genes for which some sequence information is available, at least from related species (in this case maize), and generation of PCR-compatible markers that will be "breeder friendly".

5.5.1 The advantage of TRAP markers

There are several advantages of TRAP over other DNA marker techniques. First, it combines the favorable features of the RAPD (easy to perform) and the AFLP (highly informative) methods. Second, TRAP explores the bioinformatics tools to design primers against known sequences of putative genes, while RAPD and AFLP are generated by random anonymous sequences Taking advantage of the sequence information for candidate genes in designing fixed primers, TRAP was expected to generate at least modest portion of trait specific markers. It was preferred over other marker systems, as it does not have much of the inherent limitations associated with RAPD, AFLP, RFLP and SSR markers. The RAPD marker system is known to have low repeatability due to the unpredicted behaviour of the short primers used in PCR reactions. Even though AFLP is efficient in producing multiple polymorphic fragments from a single PCR reaction, the extensive pre-PCR sample processing makes it laborious and less attractive. Expressed sequence tagged (EST) markers are also promising, but our initial attempt to get such markers using the designed fixed primer pairs (forward and reverse EST primers) designed for the respective ESTs gave monomorphic fragments. SSR markers are the most widely used marker system for plant breeding because they are user friendly, highly polymorphic and locus-specific. However, their use for rapid whole genome mapping is hampered by the large number of PCR reactions required for generating data for a

sufficiently large number of marker loci and the often huge initial cost involved in SSR development. Finally, all of these PCR-based methods are faster than hybridization-based RFLP markers, but the latter is still the standard for comparative mapping across more distantly related species. With this back drop, this study attempted to develop TRAP makers associated with genomic regions contributing to salinity tolerance and to assess their utility for mapping this trait in pearl millet.

5.5.2 Optimization of TRAP technique for pearl millet

Fixed primers were designed targeting the GR, SOD and P5CS gene families of pearl millet. PCR conditions were optimized for the fixed primers in combination with a set of arbitrary primers. During the initial PCR optimizations, it was observed that arbitrary primers could behave like RAPD primers to amplify fragments in conjunction with themselves. Hence, the fixed primer concentration in the reaction mixtures was increased thrice to that of the arbitrary primer to minimize the amplifications primed by arbitrary primer alone. The initial annealing temperature for the first five cycles was set at 35°C. The rationale behind using this temperature was that primer annealing to the DNA template depends on the matching-level of the two sequences, and amplification efficiency is determined by the effectiveness of primer binding to the template DNA. The low initial annealing temperature ensured the binding of arbitrary primer to template sites with even a partial match in the target DNA sequence. The annealing temperature was then increased for the subsequent 35 cycles to 50°C. This temperature change ensured that DNA products amplified in the initial five PCR cycles were efficiently and consistently amplified in exponential fashion during the remaining PCR cycles. If the annealing temperature was kept at 35°C for all the cycles then it would have resulted in bands of poor reproducibility (Li and Quiros, 2001). Increasing the initial annealing temperature (for the first five cycles) led to higher specificity and fewer amplified fragments. This was also observed by Liu *et al.* (2005).

The TRAP-amplified fragments were highly repeatable except for a few weak bands in certain combinations, which might have been resolved better if

autoradiography or fluorescence-based methods of detection had been used instead of silver staining. The number of fragments produced by TRAP primer combinations in this study was less than reported by Hu and Vick (2003) and Liu et al. (2005), who used fluorescence-based detection systems. Nevertheless, the strong bands were highly reproducible, easily scorable, and posed no problems for scoring on silver-stained PAGE gels. It was observed that in some mapping population progenies, the strong bands were weaker in intensity than expected, but still they were easily scorable. This behaviour might be mainly because the primers got distributed to several other competing loci where amplifications were also made, thus reducing the primer availability at the locus chosen for scoring and finally resulting in unexpectedly low PCR product band intensity for the target due to decreased amplification at that locus.

5.5.3 TRAP marker analysis

Among the TRAP primer pairs designed for the three gene sequences (GR, SOD and P5CS), those producing unique bands for both parents (Tift $23D_2B_1$-P1-P5 and WSIL-P8) were preferred for amplification of DNA from the mapping population progenies, as this minimizes the likelihood of mis-scoring tracks with low PCR product levels. Further, such unique bands for both parents could potentially be converted to co-dominant markers, provided they map at same place or have very tight linkage between them. Out of 68 polymorphic TRAP markers identified, 4 TRAP marker pairs (*viz.*, S2A7.630 and S2A7.610, S3A4.530 and S3A4.510, PA15.290 and PA15.280 and PA15.165 and PA15.160), appeared to be segregating co-dominantly in the mapping population. Each pair is likely to represent co-dominant marker alleles for a single locus as they map to the same place and exhibited banding patterns on the PAGE gels just like co-dominant SSR markers (i.e. intense bands when present alone in the individual and less intense bands when both bands are present in the individual). However, this can only be confirmed after cloning and sequence analysis. Liu et al. (2005) also noticed a few TRAP markers that exhibited co-dominant inheritance in wheat.

Each TRAP PCR reaction generated 15-25 potential TRAP marker bands, which significantly varied in their intensities. The sizes of most of the amplified

fragments ranged from 130bp to 1590 bp. A total of 68 polymorphic TRAP markers were identified from 11 fixed-random primer combination reactions, for an average of ~6 easily scorable polymorphic TRAP markers for each TRAP primer pair used. The polymorphism level detected was low compared to that reported by Liu et al. (2005), Hu and Vick (2003), Alwala et al. (2003, 2006), and Arro (2005), who got an average of more than 24 polymorphic markers per reaction. This might be due to use of a less effective detection system (silver staining) in the present study, or could be attributed to the low level of polymorphism available between the parents of this mapping population compared to those used by Liu et al. (2005). However, these results are comparable to a parallel study in our lab for developing TRAP markers for drought tolerance and stover quality in a well-characterized and highly polymorphic pearl millet mapping population (Rajaram, 2005). This study and that of Rajaram (2005) together are the first time that the silver-staining technique is being used for visualization of TRAP markers. Our results indicated that the TRAP markers provide a rapid and powerful technique for fingerprinting pearl millet for agronomically important traits like salinity tolerance, but suggest that more sensitive PCR product visualization procedures may be needed to more effectively exploit the potential of this marker system.

These TRAP markers were analyzed for segregation distortion using a chi-square test and 22 and 7 out of the 68 TRAP markers were found segregating according to the expected Mendelian ratio of 3:1 or 1:3, respectively. When the TRAP markers were tested for segregation into 1:1 ratio, 19 TRAP markers were found segregating with no significant distortion. The segregation of these nineteen TRAP markers into 1:1 ratio instead of the Mendelian 3:1 ratio may be attributable to the fact that the mapping population size was quite low in this case (only 97 genotypes) and the number of leaf samples collected for DNA extraction per genotype was also less (25-30). A larger population consisting of about 200-500 progenies, has been considered better for DNA marker studies and linkage analysis (Young, 2001; Beavis, 1994).

5.5.4 Construction of linkage map

In this investigation, we used TRAP and RFLP marker data to generate a genetic linkage map in an $F_{2:4}$ mapping population suitable for salinity tolerance QTL detection. Our goal was to exploit the advantages of TRAP markers with the premise that they could be used to generate an abundance of marker loci scattered throughout the genome and for this the previously generated RFLP markers (Liu *et al*, 1994a,b) were used to assign TRAP markers to specific linkage groups and draw comparisons with previously published pearl millet marker-based genetic linkage maps. This strategy proved efficient and led to the construction of a map containing 102 (52 RFLP and 50 TRAP) loci in a short time. Of 64 TRAP markers (4 marker pairs reduced to single co-dominant loci), 50 were mapped into the framework linkage map along with 54 RFLP markers, which were generated previously for the (Tift $23D_2B_1$-P1-P5 × WSIL-P8)-based pearl millet mapping population (Liu *et al.*, 1994a,b). The linkage analysis of the new TRAP markers in combination with previously mapped RFLP markers, helped to assign the newly developed TRAP markers into specific linkage groups so that they can be placed on the pearl millet consensus map. As TRAP markers are dominant in nature, the use of co-dominant markers in mapping analysis helps to minimize the discrepancies expected to arise due to dominant markers existing in coupling and repulsion linkage phase in the F_2 mapping population.

The TRAP markers generated in this study were distributed across all seven linkage groups of pearl millet [7, 10, 14, 3, 9, 6 and 1 TRAP marker(s) respectively, in LG1, LG2, LG3, LG4, LG5, LG6 and LG7] and the total map length was 1517.7cM (Kosambi units). Each linkage group had at least one TRAP marker and hence it appears that this marker system has sufficient potential to cover the whole genome and fill gaps in the RFLP- and SSR-based pearl millet linkage map, as previously observed in wheat (Liu *et al.*, 2005). The TRAP protocol not only efficiently produced markers in target regions of our interest as in the cases described above, but they might have also produced markers mapping to other regions. There could be three reasons for this: one is that other loci might be a member of homologous gene families to which the targeted candidate gene belongs and hence might have a complementary

sequence to that of the fixed primer used. The second reason is presumably due to the low (35°C) initial annealing temperature used in the TRAP PCR protocol, which allows low annealing specificity and the subsequent amplification of large number of fragments. However, increase in annealing temperature to 50°C for 35 cycles, after the initial 5 PCR cycles at 35°C, reduces the potential for non-specific amplification. The third reason is that the random primer may amplify in conjunction with itself (Liu *et al.*, 2005), effectively behaving like a long-primer RAPD marker. However, the third reason could be ruled out in our studies as the arbitrary-arbitrary primer combination could hardly amplify any fragments within the size range of 150–2000 bp. This is one of the two first reports on the construction of linkage maps in pearl millet using TRAP markers. TRAP markers are very efficient for rapidly generating a large number of markers scattered across the genome, which allow fragmented linkage groups to be joined and many larger marker intervals to be filled.

5.5.4.1 Comparison with previously published maps

It is difficult to make extensive comparisons of marker orders between our map and previously published maps because the majority of our markers were generated by the TRAP protocol based on primer sequences developed for the SOD, GR and P5CS gene sequences, which were not previously been used to construct pearl millet genetic maps. However, previously published RFLP-based genetic linkage map of pearl millet constructed by Liu *et al.* (1994b) and uodated by Devos *et al.* (2000), Yadav *et al.* (2002, 2004), Breese *et al.* (2002), and Qi *et al.* (2006), allowed us to draw some comparisons with RFLP loci on our map. Of the RFLPs that we used for mapping, 52 were found common with those on the map presented by Liu *et al.* (1994b). Most of them detected loci on linkage groups similar to those reported by Liu *et al.* (1994a,b) and the order of common markers along our maps agreed well with the previous maps. Therefore, we assume that our linkage group assignments are correct. However, it would be necessary to assess these TRAP markers on a linkage mapping population having better anchored marker saturation on the linkage groups (especially LG3 and LG7) before these TRAP markers can confidently be assigned to pearl millet genome.

Hence, we believe that TRAPs markers are very efficient compared to other marker systems. First, the TRAP technique generates a reasonably large number of markers per PCR reaction, comparable to the AFLP technique. Second, the TRAP technique does not require extensive pre-PCR processing of templates, as does the AFLP technique. Instead, total genomic DNA is used as template, thereby making TRAP markers as user-friendly as SSR markers, but much more efficient in terms of the number of segregating marker bands generated per PCR reaction. Although there is some uncertainty as to the nature of TRAP-amplified fragments, we found that TRAP markers are highly repeatable and more cost-effective for filling groups than SSR markers because significantly more loci could be assigned with a single PCR reaction (Liu *et al.*, 2005).

5.5.5 QTL analysis

There is considerable evidence to support the view that salt tolerance and its sub-traits are determined by multiple QTLs and that both additive and dominance effects are important in the inheritance of many of the traits associated with salt tolerance (Fooland, 2004; Flowers, 2004; Gregorio *et al.*, 2002). Genetic analysis in *Arabidopsis* by crossing the most salt-tolerant accessions with the most salt-sensitive ones suggested that the salt tolerance during germination was under polygenic control (Quesada *et al.*, 2002).

5.5.5.1 Phenotypic data analysis

F_2 populations are commonly utilized in initial assessment of the inheritance of phenotypic traits in crops. Unlike a DH or RIL population, individual progenies in the segregating F_2 population are expected to be heterozygous at 50% of their loci, and F_3 individuals derived by selfing a single F_2 individual are expected to differ in their genetic background at these loci. Due to non-availability of F_2 plants (the F_2 plants having been RFLP genotyped in the early 1990s and advanced by two generations of selfing without selection to produce $F_{2:4}$-self bulks to produce seed samples for use in downy mildew resistance screens), in our investigation, small samples from $F_{2:4}$ progenies were used for phenotypic analysis, and DNA extracted from the same individuals provided the material

for TRAP marker genotypic analysis and construction of the genetic map. Such an early generation mapping population, segregating for the germination- and early seedling growth-related traits, was identified that was based on the cross between salt-tolerant line Tift $23D_2B_1$-P1-P5 and salt-sensitive WSIL-P8. Phenotypes of the $F_{2:4}$ progenies were determined by measuring the germination percentage, and shoot and root lengths of small samples of 10-day old seedlings under saline and non-saline environments. A similar phenotypic assessment of a salinity tolerance mapping population was reported in wheat for F_2 and $F_{2:3}$ populations assessed for low Na^+ uptake trait and salt tolerance (Munns *et al.*, 2002). Analysis of variance (ANOVA) revealed significant differences between the (Tift $23D_2B_1$-P1-P5 × WSIL-P8)-based pearl millet $F_{2:4}$ mapping population progenies for seed germination percentage, and lengths of shoots and roots. Significant salinity level × genotype interaction effects ($P<0.05$) were observed also for seed germination indicating that the progenies differed in their salinity tolerance during germination.

5.5.5.2 QTL mapping

Combining the marker data set with the phenotypic data set and the linkage maps permitted evaluation of the ability of the map to detect QTLs for salt stress tolerance. We tested the utility of our map for detecting QTLs associated with agronomically important traits using germination, shoot and root lengths in saline and non-saline media, as examples. QTL analysis revealed that salt tolerance for seed germination, as well as for shoot and root lengths of 10-day old seedings as measurements, has potentially valuable QTLs associated with TRAP markers based on sequences of genes for enzymes with antioxidant activity.

Preliminary QTL analysis by CIM (Composite Interval Mapping) using PlabQTL version 1.2 (Utz and Melchinger, 2003), revealed a putative major QTL for relative shoot length (Rel_SL) that was flanked by TRAP marker S2A7.630 on LG6. This QTL locus had an LOD score of 5.18 and explained 24.7% of the total adjusted phenotypic variance (26.8%) for the mapping population progeny ratios of shoot length under saline conditions to that in the non-saline control treatment. Results indicate that the favourable allele for this QTL increased

relative shoot length under stress conditions by 18% and was inherited recessively from the salt-tolerant parent, Tift $23D_2B_1$-P1-P5. Another significant QTL, for shoot length itself of 10-day old seedlings grown in the 150mM NaCl treatment (SL_150), was detected on LG6 and was flanked by this TRAP marker. This second QTL had an LOD of 3.52 and accounted for 15.6% of the total adjusted phenotypic variance for this trait. The favourable allele for this QTL was also inherited recessively from the salt-tolerant parent. These two QTLs, which likely represent a common single gene, appear to overlap at TRAP marker S2A7.630, so this chromosomal region appears likely to be involved in the control of salt tolerance of shoot length in young pearl millet seedlings. Genetic analysis of salt tolerance in pearl millet suggests that the effect of these two QTLs (Rel_SL and SL_150) is large enough to be detected in saline environments. Several additional QTL candidates governing root and shoot lengths under saline conditions were detected and are reported as preliminary QTLs associated with salinity tolerance in pearl millet. Most of these QTLs have small effects and are therefore less likely to be reliable. The smaller the effect of QTLs, the more difficult for it is for them to be detected (Kearsey and Farquhar, 1998). Hence, these loci need to be confirmed using a proper RIL mapping population based on cross (Tift $23D_2B_1$-P1-P5 × WSIL-P8), which could be assessed for other related phenotypic traits in future studies. Analysis of the genetic control of salt tolerance were performed in several crops, including wheat (Dubcovsky *et al.*, 1996; Semikhodskii *et al.*, 1997; Munns *et al.*, 2002), barley (Ellis *et al.*, 1997; Mano and Takeda, 1997), and most extensively in rice (Lee *et al.*, 2006; Flowers *et al.*, 2000; Prasad *et al.*, 2000; Koyamo *et al.*, 2001; Lin *et al.*, 1998, 2004; Gong *et al.*, 1999), the present work probably being the first report of salt tolerance in pearl millet. Interestingly, QTL analysis of plant responses to salt stress has often revealed relatively small numbers of major QTLs (Yeo and Flowers, 1990; Zhang *et al.*, 1995; Gong *et al.*, 1999).

Physiological traits associated with salt tolerance in rice were complex and controlled by a few major QTLs. QTLs for salinity tolerance associated morphological traits like germination percentage, root length, etc., have been mapped in rice (Prasad *et al.*, 2000), but there were no QTLs identified for shoot length in that study. In anticipation of its significant contribution, the pair of

QTLs identified on linkage group 6 in our studies may contain a major gene that contributes to salt stress tolerance during the early seedling stage of growth in pearl millet. However, this needs to be confirmed by conducting field trials in saline soils for two or three seasons to test if the QTLs confer tolerance that is stable across seasons (and growth stages). Chromosome substitution at these loci can be done during the course of fine-mapping and marker-assisted backcrossing of this region of linkage group 6 from Tift $23D_2B_1$-P1-P5 into the genetic background of disease resistant, salinity-sensitive lines such as WSIL-P8 and ICMB 90111 to produce more agronomically desirable salt tolerant hybrid parental lines.

Although there are no previous reports for QTLs identified in $F_{2:4}$ populations, QTL analyses for rice salt tolerance were reported using RILs, DH lines and $F_{2:3}$ progenies (Lee *et al.*, 2006; Gong *et al.*, 1999; Koyama *et al.*, 2001; Lin *et al.*, 1998; 2004; Zhang *et al.*, 1995). In the present study, we identified two new QTLs related to the salt tolerance of seedling shoot growth of pearl millet. Zhang *et al.* (1995) and Gong *et al.* (1999) detected QTLs for salt tolerance on rice chromosomes 1 and 7, respectively, and Lin *et al.* (1998) also detected a QTL on chromosome 5 with a small effect on the length of survival of seedlings under saline conditions. Koyama *et al.* (2001) identified ten QTLs for five shoot traits related to salt tolerance in rice, such as Na^+ uptake (one QTL) and K^+ uptake (two QTLs), Na^+ concentration (two QTLs) and K^+ concentration (two QTLs), and Na^+:K^+ ratio (two QTLs). Lin *et al.* (2004) detected five QTLs for four traits associated with salt tolerance in rice roots, and three QTLs for three traits in shoots, but these QTLs did not occupy the same map locations.

The comparative genetic mapping between pearl millet and rice (Devos *et al.*, 2000) revealed that linkage group 6 of pearl millet (2n=2x=14; C=2.4pg) is largely homologous to foxtail millet (2n=2x=18; C=0.45pg) chromosome V and rice (2n=2x=24; C=0.4pg) chromosome 1. A major QTL region for domestication traits on foxtail millet chromosome V was found orthologous to regions of rice chromosome 1, pearl millet linkage group 6, and maize chromosomes 3 and 8, all of which carry genes controlling shattering ability (Paterson *et al.*, 1995; Poncet *et al.*, 2002). Interestingly, QTLs for major traits concerned with salt

tolerance have been frequently detected on rice chromosome 1 (Gong *et al.*, 1999; Koyama *et al.*, 2001; Lin *et al.*, 2004), while the two QTLs identified for salt tolerance in pearl millet from our studies lie on homologous linkage group 6. However, it is difficult to make detailed comparisons, because, for the most part, different sets of DNA markers were used in these studies and this portion of the pearl millet linkage map is poorly saturated with marker loci. None-the-less, this information might be useful in guiding future research to compare the genetic regions conferring salt tolerance across these two species, provided that enough loci can be detected using common markers. Further, in order to compare the map locations of QTLs determined by independent studies, it will be necessary to integrate several independent linkage maps of DNA markers into one map.

From our studies, we hoped to identify new sources of salinity tolerance among elite breeding materials and to identify the first QTLs for salt tolerance in pearl millet. These QTLs for detected as a result of genetic recombination in a cross between a salinity-tolerant line and a salinity-sensitive line. The detection of two QTLs (that may in fact represent a single gene of relatively large effect) associated with salt tolerance provides important information for a future functional analysis of pearl millet salt tolerance, because they were identified in an $F_{2:4}$ mapping population derived from two parents with differential responses to salinity. The molecular markers that are nearest to these QTLs (M202 and S2A7.630) may also be useful for MAS in an applied pearl millet breeding program aimed at developing parental lines of hybrid cultivars with high levels of salt tolerance. In addition, the salt-tolerant lines identified may be used as donor parents for breeding salt tolerant pearl millet. Thus, the application of QTL mapping for salt tolerance may greatly facilitate improvement of salt tolerance in pearl millet.

Finally, it is concluded that the TRAP technique is a relatively high-throughput PCR-based marker system, which offers a potentially inexpensive means for preliminary evaluation of candidate genes during development of near-perfect selectable markers for species with limited sequence information such as pearl millet. The development of a TRAP-marker based genetic linkage map of pearl

millet also allowed the partitioning of the quantitative variation of salt stress effects associated with defined chromosomal locations. In summary, modern molecular techniques offer new approaches to improving salt tolerance of crops; identifying physiological traits that are components of salt tolerance and genetic regions associated associated with these. Thus they provide tools for improving our understanding of the mechanisms of salinity tolerance. By comparing different genes and genetic combinations, researchers —especially breeders — will further be able to advance the field more quickly and develop salt-tolerant germplasm much more efficiently than has here-to-for been possible.

SUMMARY AND CONCLUSIONS

CHAPTER 6

SUMMARY AND CONCLUSIONS

Salt stress adversely affects crop productivity. Crop plants take up salts from irrigation water, and from soil made saline by evaporation of irrigation water. Because water use efficiency is often higher in salt tolerant plants than in salt susceptible plants, improving salt tolerance in food crops could have a major impact on agriculture by allowing cultivation with lower amounts and lesser quality of water. By reducing the water uptake of roots, salts cause ionic, osmotic and nutrient stresses. Cellular responses to these stresses, and subsequent secondary stresses, such as oxidative stress, add to the complexity of the salt tolerance trait. At the physiological level, salt tolerant plants exhibit a wide range of mechanisms that include exclusion, compartmentalization and secretion of salts. Although accumulation of Na^+ and Cl^- aid osmotic and water potential adjustment of cells, they increase the risk of long-term ion toxicity, if not compartmentalized appropriately, exported, or secreted. Osmotic adjustment has also been identified as a major trait of interest and is associated with the whole plant response to salt stress. Hence, an understanding of how tolerant crop species gradually adapt to salt stress should enable the development of additional salt tolerance traits.

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is one of the very few crop species that show moderate levels of salt tolerance and is a diploid cereal belonging to *Poaceae* family. It is one of the two most widely cultivated drought-tolerant C_4 cereals grown under rainfed and dry-land conditions in drought-prone regions of the tropics and subtropics. Pearl millet is especially important as a staple food grain, and source of feed and fodder for livestock, in the marginal agricultural production environments of Africa and South Asia that are home to hundreds of millions of the world's poorest crop-livestock producers. Besides soil infertility and drought, salinity is the most important abiotic constraint to production of this crop, and is a major contributor to the instability of its grain and fodder yields. This species can thus be a good model to understand salinity

and related osmotic stress tolerance mechanisms. Initial phenotypic assessment of salinity tolerance in pearl millet can provide useful data to identify tolerant lines that can then be used in identification of the mechanisms conferring their salinity tolerance.

Molecular markers are a promising tool to help us understand the genetic control of salt tolerance as well as to follow the introduction of important genomic regions for tolerance into susceptible genotypes by marker assisted selection (MAS). The expression of salt stress induced genes is an essential part of tolerance mechanisms, but for many genes with salt stress-responsive expression, no direct function in tolerance was clearly demonstrated. Consequently, mapping the location of such candidate genes near or within QTLs involved in tolerance and adaptation to saline conditions could give some information on their role. QTL mapping and subsequent marker-assisted backcrossing to improve salt tolerance of pearl millet cultivars is a promising aspect for the crop improvement in future for this 'orphan' crop with a dearth of markers.

Taking cognizance of this background information, the present work had been taken up with the following objectives:

* **To screen a set of genetically diverse pearl millet inbred lines for their salinity tolerance levels.**

* **To assess possible mechanisms of salinity tolerance in pearl millet.**

* **To measure the antioxidant enzyme activity responses to salinity stress in salt-tolerant and -sensitive lines of pearl millet.**

* **To develop TRAP (Target Region Amplified Polymorphism) markers for salinity tolerance in pearl millet.**

* **To check the validity of molecular markers identified in the course of the study.**

* **To identify QTLs for salinity tolerance in mapping populations of pearl millet.**

The aim of this study was to assess opportunities for using existing pearl millet mapping populations (Hash and Witcombe, 1994; Hash *et al.*, 2001) and other pearl millet genetic stocks available at ICRISAT to map TRAP (Target Region Amplified Polymorphism) markers for genomic regions contributing to salinity stress tolerance. The TRAP markers thus developed and mapped may find use in genome characterization, tagging desirable genes, and high-throughput mapping of pearl millet populations.

6.1 Screening for salinity stress and possible mechanisms of salt tolerance in pearl millet

Screening for salt-tolerant pearl millet germplasm is important to determine whether there is a genetic basis for observed variation in response to salinity stress that can be exploited for selection and breeding purposes. Although field screening for salt tolerance has the advantage of testing germplasm under natural conditions, it is less efficient and more expensive than screening under controlled conditions. In the present study, pearl millet inbred lines were screened for salt stress tolerance in soil-free *in vitro* environment to reduce the complexity of genotype × environment interactions. Salinity stress tolerance during germination and early seedling growth, which is critical to crop establishment in saline soil conditions was emphasized.

- For this purpose, a series of experiments were conducted in which seeds of 28 pearl millet inbred lines obtained from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India were tested for salt stress tolerance across salinity levels ranging from 50 mM to 200 mM NaCl. The highest threshold level of NaCl concentration for germination of pearl millet lines was found to be 150 mM.

- Preliminary screening for salinity tolerance in pearl millet was carried out by germinating the seeds of 28 different pearl millet inbred lines on filter-paper boats in a balanced nutrient solutions (Hoagland and Arnon, 1938)

of pH 6.7 at 20°C, containing four different concentrations of NaCl (0 mM, 75 mM, 100 mM, and 150 mM). They were grown for 10 days to monitor morphological and physiological changes in reponse to salinity. We were successful in characterizing these lines with regard to their response to salt stress.

Based on their differential ability to maintain high germination levels and seedling growth at 75 mM, 100 mM, and 150 mM NaCl, seven of the pearl millet inbred lines were categorized as sensitive (ICMB 90111, PRLT 2/89-33, P1449-2-P1, Tift 238D_1-P152, 81B-P6, WSIL-P8 and ICMP 85410-P7), fifteen as moderately tolerant, and five as highly tolerant (Tift 23D_2B_1-P1-P5, ICMB 841-P3, P310-17-Bk, ICML 22, and ICMB 95333).

At higher salinity levels (100 mM & 150 mM NaCl), the differences between germination, shoot length and shoot dry weight between tolerant and susceptible lines were highly significant.

The relative reduction of shoot length and shoot dry matter (shoot dry weight, mg/seedling) at various salinity levels compared to the non-saline control treatment was less in the most tolerant lines than in the more sensitive lines.

To validate the results obtained, a statistical analysis of the data set was done using the GenStat software package. High operational heritabilities (ratio of variance attributable to the 28 inbred line seed samples to the total variance observed for a particular character) were found in the analyses of the various observer characters, indicating that the observed differences between these 28 genotypes were statistically significant.

A genotype x environment interaction analysis was also carried out to assess whether there was significant interaction between the pearl millet genotypes and the salt stressed environments for all the parameters under study. Significant interactions would suggest that genetic differences in salinity tolerance exist among the 28 pearl millet inbred lines included in this study. ANOVA revealed that despite large effects of both genotype and

environment, genotype × environment interactions were highly significant for all observed traits, indicating that the inbred line seed lots differed in their salinity tolerance during germination and early seedling growth.

Large differences were detected between members of several pearl millet mapping population parental pairs, indicating that their previously skeleton-mapped pearl millet mapping population progeny sets can be used to map genomic regions contributing to salinity tolerance during germination and early seedling growth.

Whole plant free proline levels were seen to increase with increasing salinity levels in all the pearl millet lines irrespective of their tolerance status; however, there was higher accumulation of proline in the tolerant lines. Accumulation of proline to a higher degree under salinity stress is indicative of the fact that proline acts as a cytoplasmic osmoticum and perhaps protects the proteins against denaturation.

Because salt stress affects both water relations and ionic balance in plants, the pattern of Na^+ and K^+ accumulation in whole plants were compared in each line. The Na^+ content increased in all the lines immediately after the onset of stress; however, pearl millet lines differed in their amount of Na^+ and K^+ accumulation.

Pearl millet lines showed a general increase of Na^+ content with salt stress. However, tolerant lines with K^+/Na^+ ratio >1, showed comparatively higher accumulation of K^+ than Na^+, which indicates that K^+ accumulation is one of the possible reasons for their tolerance to salinity.

Increasing magnitude of salt exposure led to an increase in the content of Na^+, K^+, Ca^{2+} and Cl^- ions in pearl millet lines subjected to short-term salt stress. However, the increase in accumulation of Na^+ and Cl^- were comparatively higher in the salt-sensitive lines, while increase in accumulation of K^+ and Ca^{2+} were much higher in the moderately tolerant and highly tolerant lines than the salt-sensitive lines.

- Qualitative and quantitative analysis was carried out for some of the antioxidative enzymes such as superoxide dismutase (SOD), glutathione S-transferase (GST), glutathione reductase (GR), catalase (CAT) and reduced glutathione content (GSH) for short term salt stress in three representative lines of pearl millet exhibiting differential responses to salt stress. Results indicated that the salt-sensitive, moderately tolerant and highly tolerant inbreds showed differences in their mechanisms of salt stress tolerance. The mechanism of NaCl stress tolerance in seedlings of sensitive line ICMB 90111 appeared to be due to increased levels of SOD whereas in 863B-P2 and 841B-P3, elevated levels of CAT, SOD, GR, GST and glutathione were observed in reponse to salt stress. Besides, an extra isoform of Mn-SOD was also detected that showed differential expression patterns in the salt-sensitive and salt-tolerant lines.

- Exposure to increasing concentrations of NaCl for 7 days, also had differential effects on the antioxidant enzymes, GSH and lipid peroxidation across the three representative lines of pearl millet. Higher lipid peroxidation levels were observed with an increase in salinity in the sensitive line ICMB 90111 than in the more tolerant lines 863B-P2 and 841B-P3. Thus it is evident that the tolerant lines possess defense mechanisms to combat the effects of lipid peroxidation at higher salinity levels.

- In a similar experiment, in two parental lines of pearl millet with extreme responses to salt stress, Tift $23D_2B_1$-P1-P5 (salt-tolerant) and WSIL-P8 (salt-sensitive), it was observed that the activities of the antioxidative enzymes showed completely different behaviour for SOD, GR, GST and glutathione, but hardly any difference in CAT. Lipid peroxidation levels were higher showing an adverse effect of salt stress in the sensitive line WSIL-P8, but the tolerant line was not much affected. The isozyme patterns for SOD also showed different responses in these two lines, especially for Mn-SOD1.

- Comparative studies for antioxidant enzymes during short-term salt treatments suggest that salt-tolerant and salt-sensitive inbreds of pearl millet possess differential oxidative components of both enzymatic and non-enzymatic machinery for scavenging reactive oxygen species generated as a result of exposure to salt stress.

- Variations in lipid peroxidation under salt stressed conditions, as measured by MDA content, indicated that the salt-tolerant lines were effective in detoxifying electrophilic and cytotoxic lipid peroxidation products.

- Protein profiles of salt-tolerant and salt-sensitive seedlings in SDS-PAGE gels following short-term salt-stress showed differences with increasing salinity. Up-regulation of few polypeptides (180kDa, 120kDa, 54kDa, 27kDa, 18kDa, 15kDa) was noticed in tolerant lines but not in salt-sensitive inbred lines. Additionally, the absence of a 25kDa protein band in the sensitive lines, while its presence in the moderately tolerant and highly tolerant lines, indicated its association to salinity tolerance.

- Exogenous application of $CaCl_2$ and GA_3 alleviated the adverse effects of salt stress on the growth of two inbred lines, ICMB 90111(salt-sensitive) and 841B-P3 (salt-tolerant). In presence of $CaCl_2$ and GA_3 individually and together, the salt sensitive line (ICMB 90111) not only germinated and grew well, but also accumulated a significantly greater quantity of proline compared to the non-saline control. Proline content in the tolerant line also increased with exogenous application of $CaCl_2$ and GA_3.

6.2 Development of molecular markers associated with salt tolerance in pearl millet

Marker-assisted selection for quantitative traits, especially with trait-specific markers, is receiving growing attention of plant breeders. The novel TRAP marker system has the potential to provide PCR-based markers for target sequence-related gene families, detecting a large number of loci from a single reaction, unlike the SSR (simple sequence repeats) marker technique, for which

primers are expensive and time-consuming to develop, and then detect only a few loci per reaction. In the present study, TRAP markers were developed using pairs of fixed primers [for genes of Δ^1-pyrroline-5-carboxylate synthetase (P5CS) and two antioxidant enzymes (glutathione reductase and superoxide dismutase)] and arbitrary primers to detect PCR product length polymorphism among pairs of pearl millet mapping population parents that express differential sensitivity to salinity during germination and early seedling growth.

- Novel TRAP markers were developed using pairs of arbitrary primers and fixed primers for genes of P5CS, GR and SOD among pairs of pearl millet mapping population parents Tift $23D_2B_1$-P1-P5 and WSIL-P8, which express differential sensitivity to salinity during germination and early seedling growth. The designed TRAP primer pairs (fixed + arbitrary primer combinations) were screened for their ability to detect polymorphism between the mapping population parents using silver-stained PAGE gels of their PCR products. Eleven TRAP PCR reactions that employed fixed EST-based primers in combination with arbitrary primers generated 68 easily scorable polymorphic bands.

- Of 68 TRAP marker polymorphisms detected using 11 combinations of fixed and arbitrary primers, 50 TRAP markers could be mapped onto an existing RFLP-based skeleton linkage map (with a total of 122 markers) of the [Tift $23D_2B_1$-P1-P5 (tolerant to salinity stress) × WSIL-P8 (sensitive to salinity stress)]-based $F_{2:4}$ pearl millet mapping population. Linkage map construction was carried out employing MAPMAKER/EXP v3.0 with the LOD threshold value kept at 3.0 and linkage distances (in cM units) calculated using the Kosambi function.

- Analyses of variance for phenotypic data sets for the mapping population progeny $F_{2:4}$ self bulks were performed using the residual maximum likelihood algorithm (ReML), which provides best linear unbiased predictions (BLUPs) of performance of the genotypes. The BLUPs of the (Tift $23D_2B_1$-P1-P5 × WSIL-P8)-based $F_{2:4}$ mapping population, along with

their genotypic data from 122 marker loci, were used for Quantitative Trait Loci (QTL) analyses.

QTL analysis revealed TRAP markers generated were associated with variation in salinity tolerance of germination and early seedling growth. Significant associations between traits and DNA-based markers indicated the linkage map positions of putative QTLs for the observed traits, which are regions of the genome where genes controlling components of salt tolerance are located.

Composite interval mapping (CIM) using PLABQTL version 1.1 detected two promising QTLs on pearl millet linkage group 6 that are associated with shoot length under saline conditions in pearl millet. Shoot length at 150 mM NaCl (SL_150) was associated with a major QTL flanking the TRAP marker S2A7.630 on LG6. This QTL explained 15.6% of the total adjusted phenotypic variance for shoot length in seedlings growing in 150 mM NaCl. Another significant QTL for Rel_SL flanking this same TRAP marker was also detected and accounted for 24.7% of the total adjusted phenotypic variance for relative shoot length.

Thus, the TRAP marker technique not only successfully generated trait-specific markers, but also provided an efficient and robust method for augmenting genetic linkage maps in pearl millet. It offers potentially an inexpensive means for preliminary evaluation of candidate genes during development of near-perfect selectable markers for species like pearl millet with limited sequence information. The maps generated from these studies will be useful for identification of QTLs associated with other agronomically important traits (including downy mildew resistance and panicle length) that are segregating in this population.

LITERATURE CITED

CHAPTER 7

LITERATURE CITED

Acar O., Turkan I. and Ozdemir F. (2001). Superoxide dismutase and peroxidase activities in drought sensitive and resistant barley (*Hordeum vulgare* L.) varieties. Acta Physiol. Plant. 104: 351–356.

Akbar M. and Yabuno T. (1977). Breeding for saline resistant varieties of rice. IV. Inheritance of delayed-type panicle sterility induced by salinity. Jpn. J. Breed. 27: 237–240.

***Alberico G.J. and Cramer G.R. (1993).** Is the salt tolerance of maize related to sodium exclusion? I. Preliminary screening of 7 cultivars. J. Plant Nutr. 16: 2289–2303.

Aldesuquy H.S. and Ibrahim A.H. (2001). Interactive effects of seawater and growth bioregulators on water relations abscisic acid concentration and yield of wheat plants. J. Agro. Crop Sci 187: 185–189.

Al-Harbi A.R. (1995). Growth and nutrient composition of tomato and cucumber seedlings as affected by sodium chloride salinity and supplemental calcium. J. Plant Nutr. 18: 1403–1416.

Alia and Pardha Saradhi P. (1991). Proline accumulation under heavy metal stress. J. Plant Physiol. 138: 554–558.

Alia and Pardha Saradhi P. (1993). Suppression in mitochondrial electron transport is the prime cause behind stress-induced proline accumulation. Biochem. Biophys. Res. Commun. 19: 54–58.

Alia, Pardha Saradhi P. and Mohanty P. (1991). Proline enhances primary photochemical activities in isolated thylakoid membranes of *Brassica juncea* by arresting photoinhibitory damage. Biochem. Biophys. Res. Commun. 181: 1238–1244.

Alia, Pardha Saradhi P. and Mohanty P (1997). Involvement of proline in protecting thylakoid membranes against free radical-induced photodamage. J. Photochem. Photobiol. 38: 253–257.

***Al-Khatib M., McNeilly T. and Collins J.C. (1994).** Between and within cultivar variability in salt tolerance in lucerne (*Medicago sative*L.). Gen. Resour. Crop Evol. 41: 159–164.

Allen S.G., Dobrenz A.K., Schonhorst M.H., and Stoner J.E. (1985). Heritability of NaCl tolerance in germinating alfalfa seed. Agron. J. 77: 99-101.

Allouis S., X. Qi, S. Lindup, M.D. Gale & K.M. Devos (2001). Construction of BAC library of pearl millet, *Pennisetum glaucum*. Theor. Appl. Genet. 102: 1200-1205.

Almansa M.S., Palma J.M., Yañez J., del Río L.A. and Sevilla F. (1991). Purification of an iron-containing superoxide dismutase from a citrus plant, *Citrus limonum* R. Free Radic. Res. Commun. 12: 319-328.

Almansouri M., Kinet J.–M. and Lutts S. (2001). Effect of salt and osmotic stresses on germination in durum wheat (*Triticum durum* Desf.). Plant and Soil 231: 243–254.

Alscher R.G. (1989). Biosynthesis and antioxidant function of glutathione in plants. Physiol. Plant. 77: 457-464.

Alscher R.G., Erturk N. and Heath L.S. (2002). Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J. Exp. Bot. 53:1331-1341.

Alwala S., Andru S., Arro J. and Kimbeng C.A. (2003). Target Region Amplification Polymorphism (TRAP) markers for sugarcane genotyping Journal of the American Society of Sugar Cane Technologists 24: 105.

Alwala S., Andru S., Arro J., Veremis J.C. and Kimbeng C.A. (2006). Target Region Amplification Polymorphism (TRAP) for assessing genetic diversity in sugarcane germplasm collections. Crop Sci 46 : 448–455.

Anand Kumar K. (1989). Pearl millet: Current status and future potential. Outlook on Agriculture 18: 46-53.

Anand Kumar K., Andrews D.J., Jain R.P. and Singh S.D. (1984). ICMA-1 (*sic*) and ICMB-1 (*sic*) pearl millet parental lines with A1 cytoplasmic-genic male-sterility system. Crop Sci. 24: 832.

Anand Kumar K., Rai K.N., Andrews D.J., Talukdar B.S., Singh S.D., Rao A.S., Babu P.P. and Reddy B.P. (1995). Registration of ICMP 451 parental line of pearl millet. Crop Sci. 35: 605.

Andersen J.R. and Lubberstedt T. (2003). Functional markers in plants. Trends Plant Sci 8: 554–560.

Andrews D.J., Rajewski J.F. and Kumar K.A. (1993). Pearl millet: New feed grain crop. p. 198–208. In: J. Janick and J.E. Simon (eds.), New crops. Wiley, New York.

Angrish A., Kumar B. and Datta K.S. (2001). Effect of gibberellic acid and kinetin on nitrogen content and nitrate reductase activity in wheat under saline conditions. Ind. J. Plant Physiol. 6: 172–177.

Anjum F., Rishi V. and Ahmed F. (2000). Compatibility of osmolytes with Gibbs energy of stabilization of proteins. Biochem. Biophys. Acta 1476, 75 84.

Appa Rao S., Mengesha M.H. and Reddy C.R. (1986). New sources of dwarfing genes in pearl millet (*Pennisetum americanum*). Theor Appl. Genet. 73: 170–174.

Appa Rao S., Mengesha M.H., Rajagopal Reddy C. and Saideswara Rao Y. (1990). Genetics of white sheath and bleached leaf mutants in pearl millet. J. Heredity 81: 159–162.

Appa Rao S., Singh S.D., Mengesha M.H., Reddy K.N. and Rao S.N. (1996). Development and characterization of genetic stocks in pearl millet (*Pennisetum glaucum*) resistant to downy mildew (*Sclerospora graminicola*). Ind. J. Agric. Sci. 66: 221–223.

Appadurai R., Raveendran T.S. and Nagarajan C. (1982). A new male sterility system in pearl millet. Ind. J. Agric. Sci. 52: 832 834.

Apse M.P., Aharon G.S., Snedden W.A. and Blumwald E. (1999). Salt tolerance conferred by overexpression of a vacuolar Na+/H+ antiport in Arabidopsis. Science 285: 1256–1258.

Arro J.A. (2005). Genetic diversity among sugarcane clones using target region amplification polymorphism (TRAP) markers and pedigree relationships. M.Sc. thesis. Louisiana State University, USA.

Arumuganathan K. and Earle E.D. (1991). Nuclear DNA content of some important plant species. Plant Molecular Biology Reporter 9: 208–218.

Asch F., Dingkuhn M., Dörffling K. and Miezan K. (2000). Leaf K/Na ratio predicts salinity induced yield loss in irrigated rice. Euphytica 113, 109–118.

Ashraf M. (1994). Genetic variation for salinity tolerance in spring wheat. Hereditas 120: 99-104.

Ashraf M. (1999). Breeding for salinity tolerance proteins in plants. Crit. Rev. Plant Sci. 13: 17-42.

Ashraf M. (2002). Salt tolerance of cotton: S Some new advances. Crit. Rev. Plant Sci. 21: 1-30.

Ashraf M. and Idrees N. (1992). Variation in germination of some salt tolerant and salt sensitive accessions of pearl millet (*Pennisetum glaucum* (L.) R. Br.) under drought, salt and temperature stresses. Pak. J. Agric. Res. 1:15-20

Ashraf M. and McNeilly T. (1987). Salinity effects on five lines/cultivars of pearl millet (*Pennisetum americanum* L.). Plant and Soil 103: 13-19.

Ashraf M. and McNeilly T. (1989). Effect of salinity on some cultivars of maize. Maydica 34 : 179-189.

Ashraf M. and Waheed A. (1990). Screening of local/exotic accessions of lentil (*Lens culinaris* Medic.) for salt tolerance at two growth stages. Plant and Soil 128: 167-176.

Ashraf M., Aasiya K and Khanum A. (1997). Relationship between ion accumulation and growth in two spring wheat lies differing in salt tolerance at different growth stages. J. Agron. & Crop Sci. 178: 39-51.

Ashraf M., Karim F and Rasul E. (2002). Interactive effects of gibberellic acid (GA_3) and salt stress on growth, ion accumulation and photosynthetic capacity of two spring wheat (*Triticum aestivum*L.) cultivars differing in salt tolerance. Plant Growth Reg. 36: 49-59.

Ashraf M., McNeilly T. and Bradshaw A.D. (1986). The potential for evolution of salt (NaCl) tolerance in seven grass species. New Phytol. 103: 299-309.

Azhaguvel P. (2001). Linkage map construction and identification of QTLs for downy mildew (*Sclerospora graminicola*) resistance in pearl millet (*Pennisetum glaucum* '(L.) R. Br.). PhD thesis, Department of Agricultural Botany, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai, Tamil Nadu, India. .64 pp.

Azhaguvel P., Hash C.T., Rangasamy P. and Sharma A. (2003). Mapping the d_1 and d_2 dwarfing genes and purple foliage color locus *P* in pearl millet. J. Hered. 94: 155-159.

Azhar F.M. and McNeilly T. (1987). Variability for salt tolerance in *Sorghum bicolor*(L.) Moench. Under hydroponic conditions. J. Agron. Crop Sci. 195: 269–277.

Azhar F.M. and McNeilly T. (1989). Heritability estimates of variation for NaCl tolerance in *Sorghum bicolor* (L.) Moench seedlings. Euphytica 43: 69–72.

Bagci S.A., Ekiz H. and Yilmaz A. (2003). Determination of the salt tolerance of some barley genotypes and the characteristics affecting tolerance. Turk. J. Agric. For. 27: 253-260.

Baker R.D. (2003). Millet Production (Guide A-414), New Mexico State University

Bandeoglu E., Eyidogan F., Yucel M. and Oktem H.A. (2004). Antioxidant responses of shoots and roots of lentil to NaCl-salinity stress. Plant Growth Reg. 42: 69–77.

Bandurska H. (1993). In *vivo* and in *vitro* effect of proline on nitrate reductase activity under osmotic stress in barley. Acta Physiol. Plant. 15: 83-88.

Banyal S. and Rai V.K. (1983). Reversal of osmotic stress effects by gibberellic acid in *Brassica campestris*. Recovery of hypocotyl growth, protein and RNA levels in the presence of GA. Physiol. Plant. 59: 111–114.

***Basalah M.O. (1991).** Effect of salinity on seed germination and growth of squash (*Cucurbita pepo* L.). Arab Gulf J. Sci. Res., 9: 87-97.

Basalah M.O. and Mohammad S. (1999). Effect of salinity and plant growth regulators on seed germination of *Medicago sativa* L. Pak. J. Biol. Sci. 2: 651-653.

Basten C.J., Weir B. and Zeng Z.-B. (2001). QTL Cartographer. Department of Statistics, North Carolina State University, Raleigh, NC.

Basten C.J., Weir B.S. and Zeng Z.-B. (1994). Zmap-a QTL cartographer. In: J.S.G.C. Smith, B.J. Benkel,W.F. Chesnais, J.P. Gibson, B.W. Kennedy & E.B. Burnside (Eds.), Proceedings of the 5th World Congress on Genetics Applied to Livestock Production: Computing Strategies and Software, Guelph, Ontario, Canada. Published by the Organizing Committee, 5th World Congress on Genetics Applied to Livestock Production.

Bates L.S., Waldem R.P. and Teare I.D. (1973). Rapid determination of free proline for water-stress studies. Plant Soil. 39: 205–207.

Bayuelo-Jiménez J.S., Debouck D.G. and Lynch J.P. (2003). Growth, gas exchange, water relations, and ion composition of Phaseolus species grown under saline conditions. Field Crops Res. 80: 207-222.

Bayuelo-Jimênez J.S., Graig R. and Lynch J.P. (2002). Salinity tolerance of *Phaseolus* species during germination and early seedling growth. Crop Sci. 42: 1584-1594.

Beauchamp C. and Fridovich I. (1971). Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44: 276-287.

Beavis W.D. (1994). The power and deceit of QTL experiments: lessons from comparative QTL studies. Proceedings of 49th Annual Corn and Sorghum Industry Research Conference, Washington DC, ASTA.

Beckmann J. and Soller M. (1986). Restriction fragment length polymorphisms in plant genetic improvement. Oxford Surveys of Plant Mol. Biol. Cell Biol. 3: 197-250.

Beckmann J. and Soller M. (1990). Towards a unified approach to genetic mapping of eukaryotes based on sequence tagged microsatellites. Biotechnology 8: 930-932.

Beckmann J.S. and Soller M. (1983). Restriction fragment length polymorphism in genetic improvement: methodologies, mapping and costs. Theor. Appl. Genet. 67: 35-43.

Bellinger Y. and Larher F. (1987). Proline accumulation in higher plants: A redox buffer? Plant Physiol. 6: 23-27.

Ben-Hayyim G., Faltin Z., Gepstein S., Camein A.L., Strosberg D. and Eshdat Y. (1993). Isolation and characterisation of salt associated proteins in citrus. Plant Sci. 88: 129-140.

Ben-Hayyim G., Vaedia Y. and Williams B.G. (1989). Proteins associated with salt adaptation in citrus and tomato cells: Involvement of 26Kda polypeptides. Physiol Plant. 77:332-340.

Bennett M.D. (1976) DNA amount, latitude, and crop plant distribution. Environ Exp Bot 16:93-108.

Bernstein L. (1975). Effects of salinity and sodicity on plant growth. Ann. Rev. Phytopathol. 13:295–312.

Bernstein L. and Hayward H.E. (1958). Physiology of salt tolerance. Annu Rev. Plant Physiol. 9: 25–46.

Bernstein N., Silk W.K. and Läuchli A. (1993). Growth and development of sorghum leaves under conditions of NaCl stress. Planta 191: 433–439.

Bertin I., Zhu J.H. and Gale M.D. (2005). SSCP-SNP in pearl millet - a new marker system for comparative genetics. Theor. Appl. Genet. 110: 1467-1472.

Beyer W.F. and Fridovich I. (1987). Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions Anal Biochem 161: 559-566.

Bhattacharjee R., Bramel P.J., Hash C.T., Kolesnikova-Allen M.A. and Khairwal I.S. (2002). Assessment of genetic diversity within and between pearl millet landraces. Theor. Appl. Genet. 105: 666–673.

Bidinger F.R. and Hash C.T. (2004). Pearl Millet. In: Integration of Physiology and Molecular Biology in Plant Breeding (H.T. Nguyen and A. Blum, eds.). Marcel Dekker, NewYork.

Bidinger F.R., Hash C. T., Jayachandran R. and Ratnaji Rao M. N. V. (1999). Recessive, day length-insensitive earliness to synchronize flowering of pearl millet hybrid parents. Crop Sci. 39: 1049–1054.

Bliss R.D., Platt-Aloia K.A. and Thomson W.W. (1986). Osmotic sensitivity in relation to salt sensitivity in germinating barley seeds. Plant Cell Environ. 9: 721–725.

Blumwald E. (2000). Sodium transport and salt tolerance in plants. Curr. Opin. Cell Biol. 12:431–434.

Bohnert H.J. and Jensen R.G. (1996). Strategies for engineering water-stress tolerance in plants. Trends Biotechnol. 14: 89–97

Bohnert H.J. and Shen B. (1999). Transformation and compatible solutes. Sci. Hort. 78: 237–260.

Bolarin M.C., Fernandez F.G., Cruz V. and Cuartero J. (1991). Salinity tolerance in four wild tomato species using vegetative yield–salinity response

curves. Journal of the American Society for Horticultural Science 116: 286–290.

Bor M., Özdemir F. and Türkan I. (2003). The effect of salt stress on lipid peroxidation and antioxidants in leaves of sugar beet *Beta vulgaris* L. and wild beet *Beta maritima* L. Plant Sci. 164: 77–74.

Borsani O., Valpuesta V. and Botella M.A. (2003). Developing salt-tolerant plants in a new century: a molecular biology approach. Plant Cell Tissue Organ Cult. 73: 101–115.

Botstein B., White R.L., Skolnick M. and Davis R.W. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet. 32: 314–331.

Boubaker M. (1996). Salt tolerance of durum wheat cultivars during germination and early seedling growth. Agric. Medit. 126: 32–39.

Bradford M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. Anal. Biochem. 72: 248–254.

Bramel-Cox, P.J., Anand Kumar K., Hancock J.H. and Andrews D.J. (1992). Sorghum and millets for forage and feed. *In*: D.A.V. Dendy (ed.). Sorghum and the millets. chemistry and technology. Amer. Assoc. Cereal Chem.

Bray L., Chriqui D., Gloux K., Le rudulier D., Meyer M. and Peduzzi J. (1991). Betaines and free amino acids in salt stressed *in vitro* plants and winter resting buds of *Populus tricocarpa*. Physiol. Plant. 83: 136–143.

Breese W.A., Hash C.T., Devos K.M. and Howarth C.J. (2002). Pearl millet genomics – an overview with respect to breeding for resistance to downy mildew. Pages 243–246 in Sorghum and Millets Pathology 2000 (Leslie, J.F., ed.). Ames, Iowa, USA: Iowa State Press.

Brunken J.N. (1977). A systematic study of *Pennisetum* sect. *Pennisetum* (*Gramineae*). Amer. J. Bot. 64: 161–176.

Bubeck D.M., Goodman M.M., Beavis W.D. and Grant D. (1993). Quantitative trait loci controlling resistance to gray leaf spot in maize. Crop Sci. 33: 838–847.

Budak H., Pedraza F., Cregan P.B., Baenziger P.S. and Dweikat I. (2003). Development and utilization of SSRs to estimate the degree of genetic

relationships in a collection of pearl millet germplasm. Crop Sci 43: 2284-2290.

Burdon R.H., O'Kane D., Fadzillah N., Gill V., Boyd P.A., and Finch R.R. (1996). Oxidative stress and responses in *Arabidopsis thaliana* and *Oryza sativa* subjected to chilling and salinity stress. Biochem. Soc. Trans. 24: 469–472.

Burrow M.D. and Blake T.K. (1998). Molecular tools for study of complex traits. In: Molecular Dissection of Complex Traits, (Ed. Paterson, A H), CRC Press, Boca Raton, New York, pp: 13 30

Burton G.W. (1966). Pearl millet breeding. Sols Africans 11: 39 42

Burton G.W. (1969). Registration of pearl millet inbreds Tift 23B1, Tift 23A1, Tift 23DB1 and Tift 23BA1 (Reg. Nos. PL 1, PL 2, PL 3 and PL 4). Crop Sci. 9: 397.

Burton G.W. (1977). Registration of Gahi 3 pearl millet (Reg. No. 40). Crop Sci. 17: 345-346.

Burton G.W. (1980). Registration of Tift 383 and Tifleaf Tifleaf 1 pearl millet (Reg. PL 8 and Reg. No. 60). Crop Sci. 20: 293

Burton G.W. (1981). Registration of pearl millet inbreds Tift 23DBE, Tift 23DAE and Tift 756 (PL 9 to PL 11). Crop Sci 21: 317–318.

Burton G.W. and Powell J.B. (1968). Pearl millet breeding and cytogenetics. Adv. in Agron. 20: 49–89.

Busch D.S. (1995). Calcium regulation in plant cell and its role in signaling. Ann. Rev. Plant Physiol. 46: 95 102.

Busso C.S., Liu C.J., Hash C.T., Witcombe J.R., Devos K.M., deWet J.M.J. and Gale M.D. (1995). Analysis of recombination rate in female and male gametogenesis in pearl millet (*Pennisetum americanum*) using RFLP markers. Theor. Appl. Genet. 90: 242-246.

Caetano-Anolles G., Bassam B.J. and Gresshoff P.M. (1991). DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. Biotechnology 9: 553-557.

Chakrabarti N. and Mukherji S. (2002). Effect of phytohormones pretreatment on metabolic changes in *Vigna radiate* under salt stress. J. Env. Biol. 23: 295–300.

Chang H., Siegel B.Z. and Siegel S.M. (1984). Salinity-induced changes in isoperoxidases in Taro, *Colocasia esculenta*. Phytochemistry 23: 233–235.

Chang R Y, O'Donoughue L S, and Bureau T E (2001). Inter-MITE polymorphisms (IMP): a high throughput transposon-based genome mapping and fingerprinting approach. Theor. Appl. Genet. 102: 773-781.

Chao S., Sharp P.J., Killan A. and Gale M.D. (1989). RFLP based genetic maps of homoeologous group 7 chromosomes of wheat and barley, pp. 23-24 in AFRC Institute of Plant Science and John Innes Institute Annual Report, IPSR, Norwich.

Chaudhuri M. and Wiebe H.H. (1968). Influence of calcium pre-treatment on wheat germination on saline media. Plant Soil 18: 208–216.

Cheeseman J.M. (1988). Mechanism of salt tolerance in plants. Plant Physiol. 87: 547–550.

Chen G.X. and Asada K. (1989). Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. Plant Cell Physiol. 30: 987-998.

Chittenden L.M., K.F. Schertz, Y-R. Lin, R.A. Wing and A.H. Paterson. (1994). A detailed RFLP map of *Sorghum bicolor* × *S. propinquum*, suitable for high-density mapping, suggests ancestral duplication of *Sorghum* chromosomes or chromosomal segments. Theor. Appl. Genet. 87: 925-933.

Cho Y.G., Eun M.Y., McCouch S.R. and Chae Y.A. (1994). The semidwarf gene, sd-1, of rice (Oryza sativa L.). II. Molecular mapping and marker-assisted selection. Theor. Appl. Genet. 89: 54–59.

***Chopra R.N., Nayar S.L. and Chopra I.C. (1986).** Glossary of Indian Medicinal Plants (Including the Supplement). Council of Scientific and Industrial Research, New Delhi.

Chowdari K.V., Davierwala A.P., Gupta V.S., Ranjekar P.K. and Govila O.P. (1998). Genotype identification and assessment of genetic relationships in pearl millet [Pennisetum glaucum (L.) R. Br] using microsatellites and RAPDs. Theor. Appl. Genet. 97: 154–162.

Chretein D., Guillot-Salomon T., Bahl J., Cantrel C. and Jeanpaui D. (1992). Lipid and protein changes in *Jojoba* callus under salt stress. Plant Physiol. 86: 372-380.

Çiçek N. and Çakirlar H. (2002). The effect of salinity on some physiological parameters in two maize cultivars. Bulg. J. Plant Physiol. 28 (1–2): 66–74.

Claiborne A. (1985). Catalase activity. In: R. A. Greenwald (ed.), CRC Handbook of Methods for Oxygen Radical Research, 283–284. CRC Press, Boca Raton.

Collard B.C.Y., Jahufer M.Z.Z., Brouwer J.B. and Pang E.C.K. (2005). An introduction to markers, quantitative trait loci (QTL) mapping and marker assisted selection for crop improvement: The basic concepts. Euphytica 142: 169–196.

Colmer T.D., Fan T.W.-M., Higashi R.M. and Läuchli A. (1996). Interactive effects of Ca^{2+} and NaCl salinity on the ionic relations and proline accumulation in the primary root tip of *Sorghum bicolor*. Physiol. Plant. 97: 421–424.

Comba M.E., Benavides M.P. and Tomaro M.L. (1998). Effect of salt stress on antioxidant defense system in soybean root nodules. Aust. J. Plant Physiol. 25: 665–671.

Cramer G.R. and Läuchli A. (1986). Ion activities in solution in relation to Na^{+}-Ca^{2+} interactions at the plasmalemma. J. Exp. Bot. 37 : 321–330.

Cramer G.R., Epstein E. and Läuchli A. (1991). Effect of sodium, potassium and calcium on salt stressed barley. II. Elemental analysis. Physiol. Plant 81:197–202.

Cramer G.R., Epstein E., and Läuchli A. (1990). Effects of sodium, potassium and calcium on salt-stressed barley. I. Growth analysis. Physiol. Plant. 80: 83–88.

Cramer G.R., Epstien E. and Läuchli A. (1988). Kinetics of root elongation of maize in response to short-term exposure to NaCl and elevated calcium concentration. J. Exp. Bot. 39: 1513–1522.

Cramer G.R., Läuchli A. and Epstein E. (1986). Effects of NaCl and $CaCl_2$ on ion activities in complex nutrient solutions and root growth of cotton. Plant Physiol. 81: 792–797.

Crow J.F. and Dove W.F. (1988). A diamond anniversary: the first chromosome map. Genetics 118: 389–391.

***Cyrus D.P., Martin T.J. and Reavell P.E. (1997).** Salt-water intrusion from the Mazingazi river and its effects on adjacent swamp forest at Richards Bay, Zululand, South Africa. Water SA 23: 101–108.

Dadshani S.A.W., Weidner A., Buck-Sorlin G.H., Borner A. and Asch F. (2004). QTL analysis for salt tolerance in barley. Deutscher Tropentag 2004, 5–7 October in Berlin.

deLacerda C.F., Cambraia J., Oliva M.A., Ruiz H.A. and Prisco J.T. (2003). Solute accumulation and distribution during shoot and leaf development in two sorghum genotypes under salt stress. Env. & Exp. Bot. 49: 107–120.

Delauney A. and Verma D.P.S. (1993). Proline biosynthesis and osmoregulation in plants. Plant J. 4: 215–223.

Demir Y. and Kocacaliskan I. (2001). Effect of NaCl and proline on polyphenol oxidase activity in bean seedlings. Biol. Planta. 44: 607–609.

Devos K.M. and Gale M.D. (1997). Comparative genetics in the grasses. Plant Mol. Biol. 35: 3–15.

Devos K.M., Atkinson M.D., Chinoy C.N., Lui C. and Gale M.D. (1992). RFLP based genetic map of the homoeologous group 3 chromosomes of wheat and rye. Theor. Appl. Genet. 83: 931–939.

Devos K.M., Pittaway T.S., Reynolds A. and Gale M.D. (2000). Comparative mapping reveals a complex relationship between the pearl millet genome and those of foxtail millet and rice. Theor. Appl. Genet. 100: 190–198.

Dionisio-Sese M.L. and Tobita S. (1998). Antioxidant responses of rice seedling to salinity stress. Plant Sci. 135: 1-9.

Dubcovsky J., Santa Maria G., Epstein E., Luo M.-C., Dvořák J. (1996). Mapping of the K^+/Na^+ discrimination locus *Kna1* in wheat. Theor. Appl. Genet. 92: 448–454.

Dubey R.S. and Rani M. (1989). Salinity induced accumulation of free amino acid in germinating seeds differing in salt tolerance. J. Agron. Crop Sci. 163: 236–247.

Dvora´k J., Noaman M.M., Goyal S. and Gorham J. (1994). Enhancement of the salt tolerance of Triticum turgidum L. by the *Kna1* locus transferred from the Triticum aestivum L. chromosome 4D by homoeologous recombination. Theor. Appl.Genet. 87: 872-877.

Edwards M.D., Stuber C.W. and Wendfel J.F. (1987). Molecular-marker facilitated investigations of quantitative-trait loci in maize. I. Numbers, genomic distribution and types of gene action. Genetics 116: 113-125.

Ejeta G., Hassen M.M. and Mertz E.T. (1987). *In vitro* digestibility and amino acid composition of pearl millet (*Pennisetum typhoides*) and other cereals. Proc. Natl. Acad. Sci. 84: 6016.

Ellis R.P., Forster B.P., Waugh R., Bonar N., Handley L.L., Robinson D., Gordon D.C. and Powell W. (1997). Mapping physiological traits in barley. New Phytol. 137: 149-157.

Ellman G.L. (1959). Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82: 70-77.

Ender A., Schwenk K., Stadler T., Streit B. and Schierwater B. (1996). RAPD identification of microsatellites in *Daphina*. Mol. Ecolog. 5: 437-447.

Facciola. S. (1990). Cornucopia - A Source Book of Edible Plants. Kampong Publications ISBN 0-9628087-0-9

Fadzilla N.M., Finch R.P. and Burdon R.H. (1997). Salinity, oxidative stress and antioxidant responses in shoot cultures of rice. J Exp Bot 48: 325-331.

*__Fahrendorf T., Li W., Shrrosh B.S. and Dixon R.A. (1995).__ Stress response in alfalfa (*Medicago sativa* L.) XIX. Transcriptional activation of oxidative pentose phosphate pathway genes at the onset of the isoflavonoid phytoalexin response. Plant Mol. Biol. 28: 885-900.

Falconer D.S. (1989). Introduction to quantitative genetics. 3rd Edn. Ronald Press Co., New York, pp.187-193.

FAO (1992). Crop production yearbook. FAO, Rome, Italy.

FAO (2005). Global Network on Integrated Soil Management for Sustainable Use of Salt-affected Soils. Rome, Italy: FAO Land and Plant Nutrition Management Service. http://www.fao.org/ag/agl/agll/spush

FAO and ICRISAT (1996). The world sorghum and millet economics: Facts, trends and outlook. FAO, Rome, Italy and ICRISAT, Patancheru 502 324, Andhra Pradesh, India.

*Fischer R.A. (1918). The correlations between relatives on the supposition of Mendellian inheritance. Transactions and Proceedings, Botanical Society of Edinburgh, 52: 399–433.

Flowers T.J. (2004). Improving crop salt tolerance. J. Exp. Bot. 55:307–319.

Flowers T.J. and Yeo A.R. (1986). Ion relations of plant under drought and salinity. Aust. J. Plant Physiol. 13: 75–91.

Flowers T.J. and Yeo A.R. (1995). Breeding for salinity resistance in crop plants: Where next? Aust. J. Plant Physiol. 22: 875–884.

*Flowers T.J., Hajibagheri M.A. and Clipson N.C.W. (1986). Halophytes. Quarterly Rev. Biol. 61: 313–337.

Flowers T.J., Koyama M.L., Flowers S.A., Sudhakar C., Singh K.P. and Yeo A.R. (2000). QTL: their place in engineering tolerance of rice to salinity. J. Exp. Bot. 51: 99–106.

Flowers T.J., Troke P.F. and Yeo A.R. (1977). The mechanism of salt tolerance in halophytes. Annu. Rev. Plant Physiol. 28: 89–121.

Floyd R. A. and Zs-Nagy (1984). Formation of long-lived hydroxyl free radical adducts of proline and hydroxyproline in a Fenton reaction. Biochem. Biophys. Acta. 790: 94–97.

Foolad M.R. and Chen F.Q. (1999). RFLP mapping of QTLs conferring salt tolerance during the vegetative stage in tomato. Theor. Appl. Genet. 99: 235–243.

Foolad M.R. and Jones R.A. (1993). Mapping salt-tolerance genes in tomato (Lycopersicon esculentum) using trait-based marker analysis. Theor. Appl. Genet. 87: 184–192.

Foolad M.R., Zhang L.P., and Lin G.Y. (2001). Identification and validation of QTLs for salt tolerance during vegetative growth in tomato by selective genotyping. Genome 44: 444–454.

Fooland M.R. (2004). Recent advances in genetics of salt tolerance in tomato. Plant Cell Tissue Organ Cult. 76: 101–119.

Fooland M.R. Lin G.Y., Zhang L.P. and Chen F.Q. (1999). Comparison of QTLs for seed germination under non-stress, cold stress and salt stress in tomato. Plant Breed. 118: 167–173.

Foyer C., Lelandais M., Galap C. and Kunert K. (1991). Effects of elevated cytosolic glutathione reductase activity on cellular glutathione pool and photosynthesis in leaves under normal and stress conditions. Plant Physiol. 97: 863–872.

Foyer C.H. (1993). Ascorbic acid. In Alscher R.G. and Hess J.L. (Eds.): Antioxidants in higher plants, CRC Press, Florida, USA, pp:31–58.

Foyer C.H. and Halliwell B. (1976). The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133: 21–25.

Franco O.L., Filho J.E., Prisco J.T. and Filho E.G. (1999). Effects of $CaCl_2$ on growth and osmoregulator accumulation in Nacl stressed cowpea seedlings. Rev. Bras Fisiol. Veg. 11: 145–151.

Francois L.E. and E.V. Maas (1994). Crop response and management on salt-affected soils. p. 149–181. In: M. Pessarakli (ed.) Handbook of Crop Stress, Marcel Dekker, N.Y. [reprinted in Second Edition, p. 169–201. 1999]

Francois L.E., Maas E.V., Donovan T.J. and Youngs V.L. (1986). Effect of salinity on grain yield and quality, vegetative growthand germination of semi-dwarf and durum wheat. Agron.J. 78:1053–1058.

Frary A., Nesbitt T.C., Frary A., Grandillo S., Esther van der Knaap, E., Cong B., Liu J., Meller J., Elber R., Alper K.B. and Tanksley S.D. (2000). A quantitative trait locus key to the evolution of tomato fruit size. Sci. Magazine 289: 85–88.

Fridovich I. (1986). "Superoxide dismutases." Adv. Enzymol. Relat. Areas Mol. Biol. 58: 61–97.

Fukuoka S., T. Inoue, A. Miyao, L. Monna, H.S. Zhong, T. Sasaki and Y. Minobe (1994). Mapping of sequence-tagged sites in rice by single strand conformation polymorphism. DNA Res. 1: 271–277.

Gabriel O. (1971). Analytical disc gel electrophoresis. *In* W.B. Jacoby (ed.), Methods in Enzymology, Vol. 22 Academic Press, New York, pp. 565–577.

Galton P.T. (1889). Natural inheritance. MacMillan, London.

Garcia A., Senadhira D., Flowers T.J. and Yeo A.R. (1995). The effects of selection for sodium transport and of selection for agronomic characteristics

upon salt resistance in rice (*Oryza sativa* L.). Theor. Appl. Genet. 90: 1106-1111.

Garthwaite A.J., von Bothmer R. and Colmer T.D. (2005). Salt tolerance in wild Hordeum species is associated with restricted entry of Na^+ and Cl into the shoots J. Exp. Bot. 56: 2365-2378.

Gazette of India. (2005). F.No. 17-13/2005-SD-IV, Notification 5th November 2005, S.O. 1566(E), Part II- Sec.3 (ii) pp. 5-6, Govt of India Press, New Delhi, India.

GENSTAT (1995). Genstat 5 Release 3.2, Reference Manual Supplement. Clarendon Press, Oxford, UK.

Ghareyazie B., Huang N., Second G., Bennet J. and Khush G.S. (1995). Classification of rice germplasm I. Analysis using ALP and PCR based RFLP. Theor Appl Genet. 91: 218-227.

Ghoulam C., Foursy A. and Fares K. (2002). Effects of salt stress on growth, inorganic ions and proline accumulation in relation to osmotic adjustment in five sugar beet cultivars. Environ. Exp. Bot. 47: 39 50.

Gilbert H.F., Mc Lean V. and Mc Lean M. (1990). Molecular and cellular aspects of thiol disulfide exchange, Adv. Enzym. 63: 169-172.

Girija C., Smith B.N. and Swamy P.M. (2002). Interactive effects of sodium chloride and calcium chloride on the accumulation of proline and glycinebetaine in peanut (*Arachis hypogea* L.). Environ. Exp. Bot. 47: 1-10.

Glenn E., Brown J.J. and Blumwald E. (1999). Salt-tolerant mechanisms and crop potential of halophytes. Crit.Rev.Plant.Sci. 18: 227-255.

Goas G., Goas M. and Larher F. (1982). Accumulation of free proline and glycine betaine in *Aster tripolium* subjected to a saline shock: A kinetic study related to light period. Physiol. Plant. 55: 383-388.

Gomez J.M., Hernandez J.A., Jimenez A., del Rio L.A. and Sevilla F. (1999). Differential response of antioxidative enzymes of chloroplasts and mitochondria to long-term NaCl stress of pea plants. Free Radic. Res. 31 Suppl: S11 8.

Gong J.M., He P., Qian Q., Shen L.S., Zhu L.H.. *et al.* (1999). Identification of salt-tolerance QTL in rice (Oriza sativa L.). China Sci. Bull. 44: 68-71.

Gorham J. (1993). Genetics and physiology of enhanced K/Na discrimination. In: Randall PJ, ed. The fourth international symposium on genetic aspects of plant mineral nutrition. Canberra, Australia: Kluwer Academic Publishers, 151–158.

Gorham J. (1995a). Betaines in higher plants biosynthesis and role in stress metabolism. In (RM Wallsgrove ed) "Amino acids and Their Derivatives in Higher Plants." Society for Experimental Biology Seminar Series, Vol 56, Cambridge University Press, Cambridge, pp. 173-203.

Gorham J. (1995b). Mechanisms of salt tolerance in halophytes. In: C.V. Choukr-Allah, C.V. Malcolm nad A.Hamdy (eds.), Halophytes and Biosaline Africulture, Marcel Dekker, New York, pp. 207–223.

Gorham J., Bridges J., Dubcovsky J., Dvorak J., Hollington P.A., Luo M.-C. and Khan J.A. (1997). Genetic analysis and physiology of a trait for enhanced K^+/Na^+ discrimination in wheat. New Phytol. 137: 109–116.

Gorham J., Forster B.P., Budrewicz E., Wyn Jones R.G., Miller T.E. and Law C.N. (1986). Salt tolerance in the Triticeae: solute accumulation and distribution in an amphiploid derived from Triticum aestivum cv. Chinese Spring and Thinopyrum bessarabicum. J. Exp. Bot. 37: 1435–1449.

Gorham J., Wyn Jones R.G. and Bristol A. (1990). Partial characterization of the trait for enhanced K^+-Na^+ discrimination in the D genome of wheat. Planta 180, 590-597.

Gorham J., Wyn Jones R.G. and McDonnell E. (1985). Some mechanisms of salt tolerance in crop plants. Plant and Soil 89: 15–40.

Gossett D.R., Banks S.W., Millhollon E.P., Banks S.W. and Lucas M.C. (1996). Antioxidant response to NaCl stress in a control and a NaCl tolerant cotton cell line grown in the presence of paraquat, buthionine sulfoximine, and exogenous glutathione. Plant Physiol. 112: 803–809.

Gossett D.R., Millhollon E.P. and Lucas M.C. (1994a). Antioxidant response to NaCl stress in salt-tolerant and salt-sensitive cultivars of cotton. Crop Sci. 34: 706-714.

Gossett D.R., Millhollon E.P., Lucas M.C., Banks S.W. and Marney M.M. (1994b). The effect of NaCl on antioxidant enzyme activities in callus tissue

of salt tolerant and salt sensitive cotton cultivars (*Gossypium hirsutum* L.), Plant Cell Rep.13: 498–503.

Greenway H. and Munns R. (1980). Mechanisms of salt tolerance in non-halophytes. Ann. Rev. Plant Physiol. 31, 149–180.

Gregorio G.B. (1997). Tagging salinity tolerance genes in rice using amplified fragment length polymorphism (AFLP). PhD thesis, University of the Philippines, Los Baños, Philippines, 118 pp.

Gregorio G.B., Senadhira D., Mendoza R.D., Manigbas N.L., Roxas J.P. and Guerta C.Q. (2002). Progress in breeding for salinity tolerance and associated abiotic stresses in rice. Field Crop Res. 76: 91–101.

Grene R. (2002). Oxidative Stress and Acclimation Mechanisms in Plants. The Arabidopsis Book. ©2002 American Society of Plant Biologists pp1–20

Grover A., Kapoor A., Lakshmi O.S., Agarwal S., Sahi C., Katiyar-Agarwal S., Agarwal M. and Dubey H. (2001). Understanding molecular alphabets of the plant abiotic stress responses. Curr. Sci. 80: 206–216.

Gueta-Dahan Y., Yaniv Z., Zilinkas B.A. and Ben-Hayyim G. (1997). Salt and oxidative stress: similar and specific responses and their relation to salt tolerance in citrus. Planta 203: 460–469.

Gulia S.K. (2004). QTL mapping for improvement of downy mildew (*Sclerospora graminicola* (Sacc.) J. Schroet.] resitance (DMR) in pearl millet (*Pennisetum glaucum* (L.) R.Br.). hybrid parental line ICMB 89111. Ph.D. Dissertation, College of Agriculture, Chaudhary Charan Singh Haryana Agricultural University, Hisar.

Gupta P., Varshney R., Sharma P. and Ramesh B. (1999). Molecular markers and their applications in wheat breeding. Plant Breed 118: 369–390.

Gupta P.K. and Varshney R.K. (2000). The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis on bread wheat. Euphytica 113: 163–185.

Habig W.H., Pabst M.J. and Jakoby W.B. (1974). Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 246: 7130–7139.

Hagemeyer J. (1997). Salt. In: Prasad, M.N.V. editor. Plant Ecophysiology. John-Weley and Sons, Inc.. New York.

Haley C.S. and Knott S.A. (1992). A simple regression method for mapping quantitative trait loci in line crosses using flanking markers. Heredity 69: 315–324.

Hamilton I.E.W. and Heckathorn S.A. (2001). Mitochondrial adaptations to NaCl. Complex I is protected by antioxidants and small heat shock proteins, whereas complex II is protected by proline and betaine. Plant Physiol. 126: 1266–1274.

Hampson C.R. and Simpson G.M. (1990). Effects of temperature, salt and osmotic potential on early growth of wheat (*Triticum aestivum*). I. Germination. Can J. Bot. 68: 524–528.

Hancock J.D., Bramel-Cox P.J., Smith K.R., Healy B.J., Klopfenstein C.F., and Witt M.D. (1990). Selection for increased in vitro digestibility improves the feeding value of sorghum grain. J. Anim. Sci. 90(Suppl. 1):320 (Abstr.).

Hanna W.W., Dove R., Hill G.M. and Smith R. (1991). Pearl millet as an animal feed in the US. *In*: Abstracts of technical papers, Annu. Meet. S. Branch, ASA, 18th, Fort Worth, TX. 2–6 Feb. 1991. ASA: Madison, WI. pp. 7.

Hare P.D. and Cress W.A. (1997). Metabolic implications of stress-induced proline accumulation in plants. Plant Growth Reg. 21: 79–102.

Hare P.D., Cress W.A. and van Staden J. (1998). Dissecting the roles of osmolyte accumulation during stress. Plant Cell Environ 21: 535–553

Hare P.D., Cress W.A. and van Staden J. (1999). Proline synthesis and degradation: a model system for elucidating stress-related signal transduction. J. Exp. Bot. 50: 413–434.

Hare P.D., du Plessis S., Cress W.A, van Staden J. (1996). Stress-induced changes in plant gene expression. South African J. Sci. 92: 431–439.

Harinarayana G., Anand Kumar K. and Andrews D.J. (1999). Pearl Millet in Global Agriculture. Eds. I.S. Khairwal, K.N. Rai, D.J. Andrews and G. Harinarayana. Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi, India. pp. 479–506.

Harinasut P., Poonsopa D., Roengmongkol K. and Charoensatapom R. (2003). Salinity effects on antioxidant enzymes in mulberry cultivar. Sci. Asia 29: 109–113.

Harrington H.M. and Alm D.M. (1988). Interaction of heat and salt shocks in cutured tobacco. Plant Physiol. 88: 618–625.

Hasegawa P.M., Bressan R.A. and Pardo J.M. (2000a). The dawn of plant salt tolerance genetics. Trends Plant Sci. 5: 317–319.

Hasegawa P.M., Bressan R.A., Zhu J.K. and Bohnert H.J. (2000b). Plant cellular and molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol 51: 463–499.

Hash C.T. and Bramel-Cox P. (2000). Survey of molecular marker applications. In Application of Molecular Markers in Plant Breeding. (Ed. Haussmann, B.I.G., H.H. Geiger, D.E. Hess, C.T. Hash and P. Bramel-Cox). Training manual for seminar held at IITA, Ibadan, Nigeria, 16–17 August 1999. International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India, pp. 3–12.

Hash C.T. and Witcombe J.R. (1994). Pearl millet mapping populations at ICRISAT. Pages 69–75 *In*: J. R. Witcombe and R. R. Duncan (eds.), Use of Molecular Markers in Sorghum and Pearl Millet Breeding for Developing Countries: Proceedings of an ODA Plant Sciences Research Programme Conference, 29 Mar – 1 Apr 1993, Norwich, UK. Overseas Development Administration, London.

Hash C.T., Abdu Rahman M.D., Bhasker Raj A.G., and Zerbini E. (2001). Molecular markers for improving nutritional quality of crop residues for ruminants. Pages 203–217 *in*: G. Spangenberg (ed.), Molecular Breeding of Forage Crops. Proceedings of the 2nd International Symposium, Molecular Breeding of Forage Crops, Lorne and Hamilton, Victoria, Australia November 19–24, 2000.Kluwer, Dordrecht.

Hash C.T., Bhasker Raj A.G., Lindup S., Sharma A., Beniwal C.R., Folkertsma R.T., Mahalakshmi V., Zerbini E. and Blummel M. (2003). Opportunities for marker-assisted selection (MAS) to improve the feed quality of crop residues in pearl millet and sorghum. Field Crops Res. 84: 79–88.

Hash C.T., Sharma A., Singh S.D., Thakur R.P., Bhasker Raj A.G., Nijhawan D.C., Bainiwal C.R., Gale M.D., Howarth C.J., Cavan G.P.,. Breese W.A and Witcombe J.R. (2004). Release of pearl millet hybrid HHB67

Improved: An improved downy mildew resistant version of HHB67 produced by marker-assisted selection. PSP Annual Report 2004, India.

Hash, C.T. and J.R. Witcombe. (2002). Gene management and breeding for downy mildew resistance. Pages 27 - 36 *in* Sorghum and Millets Pathology 2000 (Leslie, J.F., ed.). Ames, Iowa, USA: Iowa State Press.

Hearne C.M., Ghosh S. and Todd J.A. (1992). Microsatellites for linkage analysis of genetic traits. Trends Genet. 8: 288–294.

Hernández J.A., Corpas F.J., Gómez M., del Río L.A. and Sevilla F. (1993). Salt-induced oxidative stress mediated by activated oxygen species in pea leaf mitochondria. Physiol. Plant. 89: 103 110.

Hernández J.A., Ferrer M.A. Jimenez A., Barcelo A.R. and Seliva F. (2001). Antioxidant systems and O_2 · H_2O_2 production in the apoplast of pea leaves: its relation with salt induced necrotic lesions in minor veins. Plant Physiol. 127: 817–831.

Hernández J.A., Olmos E., Corpas F., Sevilla F. and del Río L.A. (1995). Salt induced oxidative stress in chloroplasts of pea plants. Plant Sci. 105: 151–167.

Herouart D., Van Montagu M. and Inze D. (1993). Redox-activated expression of the cytosolic copper/zinc superoxide dismutase gene in Nicotiana. Proc. Natl. Acad. Sci. USA. 90: 3108–3112.

Hill G.M. and Hanna W.W. (1990). Nutritive characteristics of pearl millet grain in beef cattle diets. J. Anim. Sci. 68:2061–2066.

Hoagland D.R. and Arnon D.I. (1938). The water culture method for growing plants without soil. Univ. Calif. Agric. Exp. Circ. 347: 1–39.

Hong B., Barg R. and Ho T.H. (1992). Developmental and organ specific expression of an ABA- and stress-induced protein in barley. Plant Mol. Biol. 18: 663–674.

Hong Z., Lakkinéni K., Zhang Z., and Vèrma D.P.S. (2000). Removal of feedback inhibition of δ^1-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiol. 122: 1129–1136.

Hoseney R.C., Andrews D.J. and Clark H. (1987). Sorghum and pearl millet, p. 397–456. In: R.A. Olson and K.J. Frey (eds.). Nutritional quality of cereal

grains: Genetic and agronomic improvement. Agron. Monog. 28. ASA, Madison, WI.

Hospital F., Moreau L., Lacoudre F., Charcosset A. and Gallais A. (1997). More on the efficiency of marker-assisted selection. Theor. Appl. Genet. 95: 1181–1189.

Howard K.W.F. and Mullings E. (1996). Hydrochemical analysis of groundwater flow and saline incursion in the Clarendon Basin, Jamaica. Ground Water 34: 801–810.

http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi

Hu J. and Vick B.A. (2003). Target Region Amplification Polymorphism: A novel marker technique for plant genotyping. Plant Mol. Biol. Rep. 21: 289–294.

Hu J., Chen J., Gulya Jr. T.J. and Miller J.F. (2004). Detecting the presence of downy mildew and genotyping sunflower host plants *In* The same PCR reaction. Proceedings Sunflower Research Workshop.

Hu J., Ochoa O.E., Truco M.J. and Vick B.A. (2005). Application of the TRAP technique to lettuce (Lactuca sativa L.) genotyping. Euphytica 144: 225–235.

Huang J. and Redmann R.E. (1995). Salt tolerance of *Hordeum* and *Brassica* species during germination and early seedling growth. Can. J. Plant Sci. 75: 815–819.

Huber W., Rustagi P.N. and Sankhla N. (1974). Ecophysiological studies on Indian arid zone plants. III. Effect of sodium chloride and gibberellin on the activity of the enzymes of carbohydrate metabolism in leaves of *Pennisetum typhoides*. Oecologia (Berl.) 15: 77–86.

Hurkman W.J. and Tanaka C.K. (1987). The effects of salt on the pattern of protein synthesis in barley roots. Plant Physiol. 83: 517–524.

Hurkman W.J., Tao H.P. and Tanaka C.K. (1991). Germin-like polypeptides increase in barley roots during salt stress. Plant Physiol. 97: 366–374.

ICAR (2006). 4 New Hybrid Varieties of Pearl Millet for Food and Fodder Security in Drought Prone Areas. 25 April 2006.

ICRISAT and FAO (1996). The World Sorghum and Millet Economies: Facts, Trends and Outlook.

Jacoby B. (1994). Mechanisms involved in salt tolerance by plants. p. 97–123. In M. Pessarakli (ed.) Handbook of plant and crop stress. Marcel Dekker, New York.

Jain S., Nainawatee H.S., Jain R.K. and Chowdhury J.B. (1993). Salt-tolerance in *Brassica juncea* L. II. Salt stress induced changes in polypeptide pattern of *in vitro* selected NaCl tolerant plant. Euphytica 65: 107–112.

Jambunathan R. (1980). Improvement of the nutritional quality of sorghum and Pearl Millet. The United Nations University Press, Food and Nutrition Bulletin, Volume 2(1), January 1980.

Jana M.K., Jana S. and Acharya S.N. (1980). Salt stress tolerance in heterogeneous populations of barley. Euphytica 29: 409–417.

Jansen R. (1993). Interval mapping of multiple quantitative trait loci. Genetics 135: 205–211.

Jansen R. and Stam P. (1994). High resolution of quantitative traits into multiple loci via interval mapping. Genetics 136: 1447–1455.

Jarman A.P. and Wells R.A. (1989). Hypervariable minisatellites: recombinators or innocent bystanders? Trends Genet 5: 367–371.

Jauhar P.P. (1981). Cytogenetics of pearl millet. Adv. in Agron. 34: 407–479.

Jeschke W.D. (1984). K+-Na+ exchange at cellular membranes, intracellular compartmentation of cations, and salt tolerance. In: R.C. Staple and G.N. Toenniesen (eds.), Salinity Tolerance in Plants: Strategies for Crop Improvement. John Wiley & Sons, New York. pp. 37–66.

Jogeswar G., Pallela R., Jakka N.M., Reddy P.S., Rao J.V., Sreenivasulu N. and Kavi Kishor P.B. (2006). Differential response of the antioxidant components of drought susceptible and tolerant varieties of sorghum during seedling growth under short-term salinity stress. Acta Physiol. Plant. (in press).

Jones D.A., Thomas C.M., Hammond-Kossack K.E., Balint-Kurti P.J., Jones J.D.G. (1994). Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. Science 266: 789–793.

Jones E.S., Breese W.A., Liu C.J., Singh S.D., Shaw D.S. and Witcombe J.R. (2002). Mapping quantitative trait loci for resistance to downy mildew in

pearl millet: Field and glasshouse screens detect the same QTL. Crop Sci. 42: 1316–1323.

Jones E.S., Liu C.J., Gale M.D., Hash C.T. and Witcombe J.R. (1995). Mapping quantitative trait loci for downy mildew resistance in pearl millet. Theor. Appl. Genet. 91: 448–456.

Jones N., Ougham H. and Howard T. (1997). Markers and mapping: we are geneticists now. New Phytol. 137: 165–177.

Joshi S., Ranjekar P. and Gupta V. (1999). Molecular markers in plant genome analysis. Curr Sci 77: 230–240.

Kalendar R., Grob T., Regina M., Suoniemi A. and Schulman A. (1999). IRAP and REMAP: two new retrotransposon-based DNA fingerprinting techniques. Theor. Appl. Genet. 98: 704–711.

Kapoor R.L., Yadav H.P., Nijhawan D.C. and Prem Sagar (1989). Diagnostic Characteristics of Pearl Millet Hybrids HHB 50, HHB 60, HHB 67 and Their Parents. Haryana Agricultural University, Hisar, Haryana, India.

Kavi Kishor P.B. (1988). Effect of salt stress on callus cultures of *Oryza sativa* L. J. Exp. Bot. 39: 235–240.

Kavi Kishor P.B. (1989). Salt stress in cultured rice cells: effects of proline and abscisic acid. Plant Cell Environ 12: 629-633.

Kavi Kishor P.B. and Mehta A.R. (1987). Changes in enzymatic activities in callus cultures of cotton. Effect of gibberlic acid. Curr. Sci. 56: 1120–1122.

Kavi Kishor P.B., Hong Z., Miao G.H., Hu C.A.A. and Verma D.P.S. (1995). Overexpression of Δ^1-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. J. Plant Physiol. 108: 1387-1394.

Kavi Kishor P.B., Sangam S., Amrutha R.N., Sri Laxmi P., Naidu K.R., Rao K.R.S.S., Sreenath Rao, Reddy K.J., Theriappan P. and Sreenivasulu, N. (2005): Regulation of proline biosynthesis, degradation,uptake and transport in higher plants: Its implications in plant growth and abiotic stress tolerance. Curr. Sci. 88: 424–438.

Kearsey M.J. and Farquhar A.G. (1998). QTL analysis in plants: where are we now? Heredity 80: 137–142.

Kebebew F. and McNeilly T. (1995). Variation in response of accessions of minor millets, *Pennisetum americanum*(L.) Lecke (Pearl Millet) and *Eleusine coracana* (L.) gaertn (Finger Millet), and *Eragrostis tef* (Zucc.) trotter (Tef), to salinity in early seedling growth. Plant & Soil 175: 311-321.

Kendall M.G. and Stuart A. (1961). The Advance Theory of Statistics. Vol. II., 3rd Edn., Charles Griffin & Co., London.

Khajeh-Hosseini M., Powell A.A. and Bingham I.J. (2003). The interaction between salinity stress and seed vigour during germination of soybean seeds. Seed Sci. & Technol, 31: 715–725.

Khan A.A., Rao S.A. and McNeilly T. (2003). Assessment of salinity tolerance based upon seedling growth response functions in maize (*Zea Mays* L.). Euphytica 131: 81–89.

Khan M.H., Singha K.L.B. and Panda S.K. (2002). Changes in antioxidant levels in *Oryza sativa* L. roots subjected to NaCl salinity stress, Acta Physiol. Plant. 24: 145–148.

Khatun S. and Flowers T.J. (1995). Effects of salinity on seed set in rice. Plant Cell Environment 18: 61–67.

Khatun S., Rizzo C.A. and Flowers T.J. (1995). Genotypic variation in the effect of salinity on fertility in rice. Plant Soil 173: 239–250.

Kingsbury R.W. and Epstein, E. (1984). Selection for salt-resistant spring wheat. Crop Sci. 24:310–315.

Kinraide T.B. (1999). Interactions among Ca^{2+}, Na^{+} and K^{+} in salinity toxicity: quantitative resolution of multiple toxic and ameliorative effects. J. Exp. Bot. 50: 1495-1505.

Kochert G. (1994). RFLP technology, pp. 8–38. In: R.L. Phillips & I.K. Vasil (Eds.), DNA-based Markers in Plants. Kluwer Academic Publishers, Dordrecht.

Kolesnikova-Allen M.A. (2001). Mapping new quantitative trait loci (QTL) for downy mildew resistance in pearl millet (Ph.D. dissertation). University of Moscow, Moscow, Russia. 266 pp.

Koornneef M. and Stam P. (2001). Changing paradigms in plant breeding. Plant Physiology 125: 156–159.

Kosambi D.D. (1944). The estimation of map distances from recombination values. Ann Eugen 12:172–175

Koyama M.L., Levesley A., Koebner R.M.D., Flowers T.J. and Yeo A.R. (2001). Quantitative loci for component physiological traits determining salt tolerance in rice. Plant Physiol. 125: 406–422.

KozlowskiT.T. (1997). Response of woody plants to flooding snd salinity. Tree Physiology Monograph No.1, Heron Publisheing , Victoria, Canada.

Kumar B. and Singh B. (1996). Effect of plant hormones on growth and yield of wheat irrigated with saline water. Ann. Agric. Res. 17: 209-212.

Kumar L.S. (1999). DNA markers in plant improvement: An overview. Biotechnology Advances 17: 143-182.

Kuznetsov V.V. and Shevyakova N.I. (1999). Proline under stress: Biological role, metabolism, and regulation. Russ. J. Plant Physiol. 46: 274–287.

Lal R.K. (1985). Effect of salinity applied at different stages of growth on seed yield and its constituents in field peas (Pisum sativum L. var. arvensis). Indian J. Agron, 30: 296-299.

Lander E.S. and Botstein D. (1989). Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121:185–99

Lander E.S., Green P., Abrahamson J., Barlow A., Daley M.J., Lincoln S.E., and Newburg L. (1987). MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Larcher W. (1980). Physiological Plant Ecology. 2nd edition. Springer-Verlag, NewYork.

Larson R.A. (1988). The antioxidants of higher plants. Phytochem. 27: 969-978.

Lazof D.B. and Bernstein N. (1999). The NaCl induced inhibition of shoot growth: the case for distributed nutrition with special consideration of calcium. Adv. Bot. Res. 29: 113–139.

Lee D.H., Kim Y.S. and Lee C.B. (2001). The inductive responses of the antioxidant enzymes by salt stress in the rice (*Oryza sativa* L.). J. Plant Physiol. 158: 737–745.

Lee G.J., Roseman A.M., Saibil H.R. and Vierling E. (1997). A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. EMBO J 3:659-671

Lee K.S., Choi W.Y., Ko J.C., Kim T.S. and Gregorio G.B. (2003). Salinity tolerance of japonica and indica rice (Oryza sativaL.) at the seedling stage. Planta 216: 1043-1046.

Lee S.Y., Ahn J.H., Cha Y.S., Yun D.W., Lee M.C., Ko J.C., Lee K.S. and Eun M.Y. (2006). Mapping of Quantitative Trait Loci for Salt Tolerance at the Seedling Stage in Rice. Mol. Cells 21: 192–196.

Levitt J. (1980). Responses of plants to environmental stresses. Vol. II: Water, radiation, salt and other stresses. 2nd edition, Academic Press, New York, USA pp.365–488.

Li G. and Quiros C.F. (2001). Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in Brassica. Theor. Appl. Genet. 103: 455–461.

Li L., Kim B.-G., Cheong Y.H., Pandey G.K. and Luan S. (2006). A Ca^{2+} signaling pathway regulates a K^{+} channel for low-K response in *Arabidopsis*. Proc. Natl. Acad. Sci. USA 103: 12625–12630.

Lin C.C. and Kao C.H. (1995). Levels of endogenous polyamines and NaCl inhibited growth of rice seedlings. Plant Grwoth reg. 17: 15–20.

Lin H.X., Yanagihara S., Zhuang J.Y., Senboku T., Zheng K.L., *et al.* (1998). Identification of QTL for salt tolerance in rice via molecular markers. Chin. J. Rice Sci. 12: 72-78.

Lin H.X., Zhu M.Z., Yano M., Gao J.P., Liang Z.W., *et al.* (2004). QTLs for Na+ and K+ uptake of the shoots and roots controlling rice salt tolerance. Theor. Appl. Genet. 108: 253- 260.

Lincoln S., Daly M. and Lander E. (1992a). Construtcting genetic maps with MAPMAKER/EXP 3.0. Whitehead Institute Technical Report, 3rd edition.

Lincoln S., Daly M. and Lander E. (1992b). Mapping genes controlling quantitative traits with MAPMAKER/QTL 1.1. Whitehead Institute Technical Report, 2nd edition.

Lincoln S., M. Daly and E. Lander (1993). Mapping genes controlling quantitative traits using MAPMAKER/QTL. Version 1.1. Whitehead Institute for Biomedical Research Technical Report, 2nd Edn.

Lindsay M.P., Lagudah E.S., Hare R.A. and Munns R. (2004). A locus for sodium exclusion (*Nax1*), a trait for salt tolerance, mapped in durum wheat. Func. Plant Biol. 31: 1105–1114.

Liu B. (1998). Statistical Genomics: Linkage, Mapping and QTL Analysis CRC Press, Boca Raton.

Liu C.J., Devos K.M., Witcombe J.R., Pittaway T.S. and Gale M.D. (1996). The effect of genome and sex on recombination rates in *Pennisetum* species. Theor. Appl. Genet. 93: 902–908.

Liu C.J., Witcombe J.R., Hash C.T., Busso C.S., Pittaway T.S., Nash M. and Gale M.D. (1994a). Construction and application of RFLP-based genetic maps in pearl millet. pp. 57–63. In: Use of molecular markers in sorghum and pearl millet breeding for developing countries: Proceedings of an ODA Plant Sciences Research Programme Conference, 29 Mar- 1 Apr 1993, Norwich, UK (Witcombe J.R. and Duncan R.R., eds.). London, UK: Overseas Development Administration.

Liu C.J., Witcombe J.R., Pittaway T.S., Nash M., Hash C.T. and Gale M. D. (1992). Restriction length polymorphism in pearl millet, *Pennisetum glaucum*. p. 233–241 *in* Complexes d'especes, flux de genes, et ressources genetiques des plantes, actes du colloque international, Paris 8-10 janvier 1992, organise en homage a Jean Pernes, Professeur a l'Universite de Paris-XI. Lavoisier-Technique et Documentation: F-94236 Cachan Cedex, France.

Liu C.J., Witcombe J.R., Pittaway T.S., Nash M., Hash C.T., Busso C.S. and Gale M.D. (1994b). An RFLP-based genetic map of pearl millet (*Pennisetum glaucum*). Theor. Appl. Genet. 89, 481–487.

Liu Z. H., Anderson J. A., Hu J., Friesen T. L., Rasmussen J. B., and Faris J. D. (2005). A wheat intervarietal genetic linkage map based on microsatellite and target region amplified polymorphism markers and its utility for detecting quantitative trait loci. Theor. Appl. Genet. 111: 782–794

Lopez F., Vansuyt G., Fourcroy P. and Case-Delbart F. (1994). Accumulation of a 22-kDa protein and its mRNA in the leaves of *Raphanus sativus* in response to salt stress or water-deficit. Physiol. Plant. 91: 605–614.

Lovato M.B., Martins P.S. and De Lemos Filho J.P. (1994). Germination of *Stylosanthes humilis* populations in the presence of NaCl. Aust. J. Bot. 42: 717–723.

Lutts S., Kinet J.M. and Bouharmont J. (1996a). NaCl-induced senescence in leaves of rice (*Oryza sativa*L.) cultivars differing in salinity resistance. Ann. Bot. 78: 389–398.

Lutts S., Kinet J.M. and Bouharmont J. (1996b). Effects of salt stress on growth, mineral nutrition and proline accumulation in relation to osmotic adjustment in rice (*Oryza sativa* L.) cultivars differing in salinity resistance. Plant Growth Regul. 19: 207–218.

Lyamichev V., Brow M.A.D. and Dahlberg J.E. (1993). Structure-specific endonucleolytic cleavage of nucleic acids by eubacterial DNA polymerases. Science 260: 778–783.

Ma S., Gong Q. and Bohnert H.J. (2006). Dissecting salt stress pathways. J Exp. Bot. 57: 1097–1107.

Maas E.V. and Grieve C.M. (1990). Spike and leaf development in salt-stressed wheat. Crop Sci. 30: 1309–1313.

Maas E.V. and Poss J.A. (1989). Salt sensitivity of wheat at various growth stages. Irrig. Sci. 10: 9–40.

Maas E.V., Hoffman G.J., Chaba G.D., Poss J.A. and Shannon M.C. (1983). Salt sensitivity of corn at various growth stages. Irrig. Sci. 4: 45–57.

Maathuis F. J. M. and Amtmann A. (1999). K^+ nutrition and Na^+ toxicity: The basis of cellular K^+/Na^+ ratios. Ann. Bot. 84:123–133.

Mace E.S., Buhariwalla H.K. and Crouch J.H. (2003). A high throughput DNA extraction protocol for tropical molecular breeding programs. Plant Molecular Biology Reporter 21:459–460.

Madamanchi N., Alscher R., Hatzios K. and Cramer C. (1994). "Acquired resistance to herbicides in pea cultivars by exposure to sulfur dioxide." Pest. Biochem. Physiol. 48: 31–40

Maiti R.K., Amaya L.E.D., Cardona S.I., Dimas A.M.O., De La Rosa-Ibarra M. and Castillo H.D.L. (1996). Genotypic variability in maize cultivars (*Zea mays* L.) for resistance to drought and salinity. J. Plant Physiol. 148: 741-744.

Malcolm C.V., Lindley V.A., O'Leary J.W., Runciman H.V. and Barrett-Lennard E.G. (2003). Halophyte and glycophyte salt tolerance at germination and the establishment of halophyte shrubs in saline environments. Plant Soil 253: 171-185.

Maniatis T., Fritsch E.F. and Sambrook J. (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Manly K.K. and Cudmore R.H. (1998). Map Manager XP. Plant and Animal Genome VI, San Diego, California, January, 1998, pp. 75.

Manly K.K., H. Cudmore Robert Jr. and Meer J.M. (2001). Map Manager QTX, cross-platform software for genetic mapping. Mamm. Genome 12: 930-932.

Mannervik B. and Danielson U.H. (1988). Glutathione transferases: structure and catalytic activity. CRC Crit. Rev. Biochem. 23: 283–337.

Mano Y. and Takeda K. (1995). Varietal variation and effects of some major genes on salt tolerance in barley seedlings. Bull Res Inst Bioresour Okayama Univ 3: 71-81.

Mano Y. and Takeda K. (1996). Genetical studies on salt tolerance at germination in recombinant inbred, isogenic, and double haploid lines of barley (Hordeum vulgareL.) Bull Res Inst Bioresour Okayama Univ 4: 79–88.

Mano Y. and Takeda K. (1997a). Diallel analysis of salt tolerance at germination and the seedling stage in barley (*Hordeum vulgare* L.). Breeding Sci. 47: 203-209.

Mano Y. and Takeda K. (1997b). Mapping quantitative loci for salt tolerance at germination and the seedling stage in barley (Hordeum vulgareL.). Euphytica 94: 263-272.

Mansour M.M.F. (2000). Nitrogen containing compounds and adaptation of plants to salinity stress. Biol. Plant. 43: 491–500.

Marklund S. and Marklund G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 47: 469–474.

Marrs K.A. (1996). The functions and regulation of glutathione-*S*-transferase in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47: 127–158.

Martinez-Cob A., Aragues R. and Royo A. (1987). Salt tolerance of barley (*Hordeum vulgare* L.) cultivars at the germination stage: Analysis of response functions. Plant & Soil 104: 53–56.

Mather K. (1949). Biometrical Genetics, Methuen, London.

Matysik J., Alia, Bhalu B. and Mohanty P. (2002). Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. Current Science 82: 525–532.

May M.J. and Leaver C.J. (1993). Oxidative stimulation of glutathione synthesis in Arabidopsis thaliana suspension cultures. Plant Physiol. 103: 621–627.

May M.J., Vernoux T., Leaver C., Van Montagu M. and Inze D. (1998). Glutathione homeostasis in plants: implications for environmental sensing and plant development. J. Exp. Bot. 49: 649–667.

McCord J.M. (2000). The evolution of free radicals and oxidative stress. Am. J. Med. 108: 652–659

McCouch S.R., Chen X., Panaud O., Temnykh S., Xu Y., Cho Y., Huang N., Ishii T. and Blair M. (1997). Microsatellite marker development, mapping and applications in rice genetics and breeding. Plant Mol Biol 35: 89–99.

Meloni D.A., Oliva M.A., Martinez C.A. and Cambraia J. (2003). Photosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. Environ. Exp. Bot. 49: 69–76.

Meneguzzo S., Navari-Izzo F. and Izzo R. (1999). Antioxidative responses of shoots and roots of wheat to increasing NaCl concentrations. J. Plant Physiol. 155: 274–280.

Meneguzzo S., Sgheeri C.L.M., Navari-Izzo F. and Izzo R. (1998). Stromal and thylakoid-bound ascorbate peroxidases in NaCl treated wheat. Physiol. Plant. 104: 735–740.

Meyer W, Michell T G, Freedman E Z, and Vilgalys R (1993). Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococus neoformans*. J Clinical Biol. 31: 274–280.

Michelmore R., I. Paran & R. Kesseli (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. USA 88: 9828–9832.

Miklas P.N., Hu J., Grunwald N.J. (2004). Potential Application Of Trap Markers For Tagging Disease Resistance Traits In Common Bean. Proceedings Of The Bean Improvement Cooperative, Oct 27-29, Sacramento, Ca, 2003. *In* Annual Report Bean Improvement Coop. 47: 79–80.

Miklas P.N., Hu J., Grunwald N.J. and Larsen K.M. (2006). Potential Application of TRAP (Targeted Region Amplified Polymorphism) Markers for Mapping and Tagging Disease Resistance Traits in Common Bean. Crop Sci. 46: 910-916

Misra N. and Gupta A. (2005). Effect of salt stress on proline metabolism in two high yielding genotypes of green gram under saline stress. Plant Physiol. Biochem. 33: 3-38.

Misra N., Gupta A.K. and Dwivedi U.N. (2006). Changes in free amino acids and stress proteins synthesis in two genotypes of green gram under salt stress. J. Plant Sci. 1: 56–66.

Mittova V., Tal M., Volokita M. and Guy M. (2002). Salt stress induces up-regulation of an efficient chloroplast antioxidant system in the salt-tolerant wild tomato species *Lycopersicon pennellii* but not in the cultivated species. Physiol. Plant. 115: 393–400.

Mohan M., Nair S., Bhagwat A., Krishna T.G., Yano M., Bhatia C.R. and Sasaki T. (1997). Genome mapping, molecular markers and marker-assisted selection in crop plants. Mol. Breed. 3: 87-103.

Morgan R.N., Wilson J.P., Hanna W.W. and Ozais-Akins P. (1998). Molecular markers for rust and pyricularia leaf spot disease resistance in pearl millet. Theor. Appl. Genet. 96: 413–420.

Morton N.E. (1955). Sequential tests for the detection of linkage. Am. J. Human Genet. 7: 277–318.

Munjal R. and Goswami C.L. (1995). Response of chloroplastic pigments to NaCl and GA_3 during cotton cotyledonary leaf growth and maturity. Agric. Sci. Digest. 15: 146–150.

Munns R. (2001). Salinity, growth and phytohomones. *In* A. Lau¨chli and U. Lu¨ttge (eds.), *Salinity: Environment – plants – molecules*, Klewer Academic Publishers, Inc., Netherlands.

Munns R. (2002). Comparative physiology of salt and water stress. Plant Cell Environ. 25: 239–250.

Munns R. and James R.A. (2003). Screening methods for salinity tolerance: a case study with tetraploid wheat. Plant and Soil 253: 201–218.

Munns R. and Passioura J.B. (1984). Effect of prolonged exposure to NaCl on the osmotic pressure of leaf xylem sap from intact transpiring barley plants. Aust. J. Plant Physiol. 11: 497–507.

Munns R. and Termaat A. (1986). Whole-plant responses to salinity. Aust. J. Plant Physiol. 13:143–160.

Munns R., Greenway H., Delane R. and Gibbs J. (1982). Ion concentration and carbohydrate status of the elongating leaf tissue of *Hordeum vulgare* growing at high external NaCl. II. Cause of the growth reduction. J. Exp. Bot. 33:574–583.

Munns R., Husain S., Rivelli A.R., James R.A., Condon A.G. (Tony), Lindsay M.P., Lagudah E.S., Schachtman D.P. and Hare R.A. (2002). Avenues for increasing salt tolerance of crops, and the role of physiologically based selection traits. Plant and Soil 247: 93–105.

Munns R., James R.A. and Läuchli A. (2006). Approaches to increasing the salt tolerance of wheat and other cereals. J. Exp. Bot. 57: 1025–1043.

Murillo-Amador B., Lopez-Aguilar R., Kaya C., Larrinaga-Mayoral J. and Flores-Hernandez A. (2002). Comparative effects of NaCl and polyethylene glycol on germination, emergence and seedling growth of cowpea. J. Agron. Crop Sci. 188: 235–247.

Naidu B.P. (2003). Production of betaine from Australian Melaleuca spp. For use in agriculture to reduce plant stress. Aus. J. Exp Agri. 43: 1163–1170.

Nayyar H., Walia D. P. and Kaistha B.L. (1995). Performance of bread wheat (*Triticum aestivum* L.) seed primed with growth regulators and inorganic salts. Ind. J. Agric. Sci. 65: 116–122.

Nelson J.C. (1997). Qgene—software for marker-based genomic analysis and breeding. Mol. Breed. 3: 239–245.

Nepolean T. (2003). Identification of QTLs for yield and its component traits and downy mildew [*Sclerospora graminicola*(L.) R. Br.].(Ph.D. dissertation). Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. 120 pp.

Neumann P. (1997). Salinity resistance and plant growth revisited. Plant Cell Environ. 20: 1193–1198.

Nikiforov T.T., Rendle R.B., Goelet P., Rogers Y.H., Kotewicz M.L., Anderson S., Trainor G.L. and Knapp M.R. (1994). Genetic bit analysis: a solid phase method for typing single nucleotide polymorphisms. Nucl. Acids Res. 22: 4167–4175.

Niu X., Bressan R.A., Hasegawa P.M. and Pardo J.M. (1995). Ion homeostasis in NaCl stress environment. Plant Physiol. 109: 735–742.

Noble C.L. and Rogers M.E. (1992). Arguments for the use of physiological criteria for improving the salt tolerance in crops. Plant Soil 146, 99–107.

Noctor G. and Foyer C.H. (1998). Ascorbate and glutathione: keeping active oxygen under control. Ann. Rev. Plant Physiol. Plant Mol. Biol. 49: 249–279.

Noori S.A. Sadat and McNeilly T. (2000). Assessment of variability in salt tolerance based on seedling growth in *Triticum durum* Desf. Gen. Resour. & Crop Evol. 47: 285–291.

Norlyn J.D. and Epstein E. (1984). Variability in salt tolerance of four triticale lines at germination and emergence. Crop Sci. 24:1090–1092.

Nuccio M.L., Rhodes D., McNeil S.D. and Hanson A.D. (1999). Metabolic engineering of plants for osmotic stress resistance. Curr. Opin. Plant Biol. 2: 128–134.

Ohta M., Hayashi Y., Nakashima A., Hamada A., Tanaka A., Nakamura T. and Hayakawa T. (2002). Introduction of a Na^{+}/H^{+} antiporter gene from *Atriplex gmelini* confers salt tolerance to rice. FEBS letters 532: 279–282.

Orita M., Iwahana H., Kanazawa H., Hayashi K. and Sekiya T. (1989). Detection of polymorphisms of human DNA by gel electrophoresis as single-

strand conformation polymorphisms. Proc. Natl. Acad. Sci. USA 86: 2766-2770.

Othman Y., Al-Karaki G., Al-Tawaha A. R. and Al-Horani A. (2006). Variation in Germination and Ion Uptake in Barley Genotypes under Salinity Conditions. World J. Agri. Sci. 2: 11-15.

Padi F.K., Devos K.M. and Gale M.D. (2001). A downy mildew resistance QTL on pearl millet LG 4 is linked to the d_2 dwarfing gene. Poster Abstract: Plant & Animal Genome IX Conference, San Diego, CA, January 13-17, 2001.

Paleg L.G., Steward G.R. and Bradbeer J.W. (1984). Proline and glycine betaine influence protein solvation. Plant Physiol. 75: 974-978.

Paleg L.G., Stewart G.R. and Starr R. (1985). The effect of compatible solutes on profiles.*In* Biosalinity in Action: Bio Production with Saline Water. Pasternak D. & San Pietro (Edn.) martimus Nijohoff Publisher, Dordrecht, pp:83.

Pandey U.K. and Srivastava R.D.L. (1995-97). Some observations on the chemical composition of rice plants grown under varying levels of salinity. Farm Sci. J. (10 12): 116-120.

Parasher A. and Varma S.K. (1988). Effect of pre-sowing seed soaking in gibberellic acid on growth of wheat (*Triticum aestivum* L.) under different saline conditions. Ind. J. Biol. Sci. 26: 473-475.

Parida A.K., Das A.B. and Mohanty P. (2004b). Investigations on the antioxidative defence responses to NaCl stress in a mangrove, *Bruguiera parviflora*: Differential regulations of isoforms of some antioxidative enzymes. Plant Growth Regul. 42: 213-226.

Parida A.K., Das, A.B. and Das P. (2002). NaCl stress causes changes in photosynthetic pigments, proteins and other metabolic components in the leaves of a true mangrove, *Bruguiera parviflora*:, in hydroponic cultures. J. Plant Biol. 45: 28-36.

Parida A.K., Das, A.B., Mohanty, P. (2004a). Defense potentials to NaCl in a mangrove, *Bruguiera parviflora*: Differential changes of isoforms of some antioxidative enzymes. J. Plant Physiol. 161: 531-542.

Paterson A.H. (1996). Making genetic maps. In: A.H. Paterson (Ed.), Genome Mapping in Plants, pp. 23–39. R. G. Landes Company, San Diego, California; Academic Press, Austin, Texas.

Paterson A.H., Lander E.S., Hewitt J.D., Peterson S., Lincoln S.E. and Tanksley S.D. (1988). Resolution of quantitative factors into Mendellian factors by using complete linkage map of restriction fragment length polymorphisms. Nature 335: 721–726.

Paterson, A.H., Lin, Y.-R., Li, Z., Schertz, K.F., Doebley, J.F., Pinson, S.R.M., Liu, S.-C., Stansel, J.W., and Irvine, J.E. (1995). Convergent domestication of cereal crops by independent mutations at corresponding genetic loci. Science 269: 1714–1718.

Patterson H.D. and Thompson R. (1971). Recovery of inter-block information when block sizes are unequal. Biometrika, 58: 545–554.

Pei Z.M., Murata Y. Benning G., Thomine S., Klüsener B., Allen G.J., Grill E. and Schroeder J.I. (2000). Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature 406: 731–734.

Penner G. (1996). RAPD analysis of plant genomes, In: P.P. Jauhar (Ed.), Methods of Genome Analysis in Plants, pp. 251–268. CRC Press, Boca Raton.

Pérez-Alfocea F. and Larher F. (1995). Effects of phlorizin and p-chloromercuribenzenesulfonic acid on sucrose and proline accumulation in detached tomato leaves submitted to NaCl and osmotic stress. J. Plant Physiol. 145: 367–373.

Pérez-Alfocea F., Estan M.T., Santa Cruz A. and Bolarin M.C. (1993). Effects of saliity on nitrate, total nitrogen, soluble protein and free amino acid levels in tomato plants. J. Hort. Sci. 68: 1021–1027.

Petit, J. F., Nicaise, M., Lepoivre, M., Guissani, A and Lemaire, G. (1996) Protection by gluthatione against the antiproliferative effects of nitric oxide. Dependence on kinetics of no release. *Biochem. Pharmacol.* 52: 205–212.

Phillips R.L. and Vasil I.K. (1994). eds. DNA Based Markers in Plants. Dordrecht, The Netherlands: Kluwer

Pickett C.B. and Lu A.Y.H. (1989). Glutathione *S*-transferases: gene structure, regulation, and biological function. Annu. Rev. Biochem. 58: 743-764.

Poncet V., Lamy F., Devos K.M., Gale M.D., Sarr A. and Robert T. (2000). Genetic control of domestication traits in pearl millet (*Pennisetum glaucum* L., Poaceae). Theor. Appl. Genet. 100: 147-159.

Poncet V., Martel E., Allouis S., Devos K., Lamy F., Sarr A., and Robert T. (2002). Comparative analysis of QTLs affecting domestication traits between two domesticated Ã— wild pearl millet (*Pennisetum glaucum* L., Poaceae) crosses. Theor. Appl. Genet. 104: 965–975.

Powell W., Machray G. and Provan J. (1996). Polymorphism revealed by simple sequence repeats. Trends Plant Sci 1: 215–222.

Prakash V. and Sastry E.V. (1992). Effect of salinity on germination and seedling growth in wheat. Ann. Arid Zone. 31: 71–72.

Prasad S.R., Bagali P.G., Hittalmani S. and Shashidhar H.E. (2000). Molecular mapping of quantitative trait loci associated with seedling tolerance to salt stress in rice (*Oryza sativa* L.). Curr. Sci. 78: 162-164.

Prioul J.-L., Quarrie S., Causse M. and de Vienne D. (1997). Dissecting complex physiological functions through the use of molecular quantitative genetics. J. Exp. Bot. 48: 1151–1163.

Qi X., Lindup S., Pittway T.S., Allouis S., Gale M.D. and Devos K.M. (2001). Development of simple sequence repeat markers from bacterial artificial chromosomes without subcloning. Biotechniques 31: 355–362.

Qi X., Pittaway T.S., Lindup S., Liu H., Waterman E., Padi F.K., Hash C.T., Zhu J., Gale M.D. and Devos K.M. (2004). An integrated genetic map and a new set of simple sequence repeat markers for pearl millet, Pennisetum glaucum. Theor. Appl. Genet. 109: 1485–1493.

Qi X., Stam P. and Lindhout P. (1996). Comparison and integration of four barley genetic maps. Genome 39: 379–394.

Quesada V., Garcia-Martinez S., Piqueras P., Ponce M.R. and Micol J.L. (2002). Genetic architechture of NaCl tolerance in *Arabidopsis*. Plant Physiol. 130: 951-963.

Rai K.N. and Rao A.S. (1998). Registration of pearl millet parental lines ICMA 89111 and ICMB 89111. Crop Sci. 38: 1412–1413.

Rai K.N., Talukdar B.S., Singh S.D., Rao A.S., Rao A.M. and Andrews D.J. (1994). Registration of ICMP 423 parental line of pearl millet. Crop Sci. 34: 1430.

Rai K.N., Thakur R.P. and Rao A.S. (1998). Registration of pearl millet parental lines ICMA 92666 and ICMB 92666 with multiple disease resistance. Crop Sci. 38: 575.

Rajaram V. (2005). Development of TRAP markers for *tb1* gene and *brown midrib* genes in pearl millet. M.Sc. Dissertation, Department of Agricultural Biotechnology, Acharya N.G. Ranga Agricultural University, Hyderabad.

Ramagopal S. (1987). Salinity stress induced tissue-specific proteins in barley seedlings. Plant Physiol. 84: 324–331.

Rani U.R. and Reddy A.R. (1994). Salt stress-responsive polypeptides in germinating seeds. J. Plant Physiol. 143: 250–253.

Rao G.G. and Rao G.R. (1981). $^{14}CO_2$ incorporation into leaves of pigeonpea and sesame under salt stress. J. Nucl. Agric. Biol. 10: 123–126.

Rao S.A. (1997). The potential for breeding *Zea mays* (L.) for saline conditions. Ph.D. thesis, The University of Liverpool, UK.

*__Redmann R.E. (1974).__ Osmotic and specific ion effect on the germination of alfalfa. Canadian J. Bot. 52: 803–808.

Rhodes D. and Hanson A.D. (1993). Quaternary ammonium and tertiary sulfonium compounds in higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44: 357–384.

Rodriguez-Navarro A. (2000). Potassium transport in fungi and plants. Biochem. Biophysiol. Acta 1469:1–30.

Rogers M.E. and Noble C.L. (1992). Variation in growth and ion accumulation between two selected populations of *Trifolium repens* L. differing in salt tolerance. Plant Soil 146: 131–136.

Rojas-Barros P., Jan C.C. and Hu J. (2005). Mapping of a recessive branching gene in RHA 271 using molecular markers. Proc. 27th Sunflower Research Forum, January 2005, Fargo, ND.

Romero-Aranda R., Soria T. and Cuartero J. (2001). Tomato plant-water uptake and plant-water relationships under saline growth conditions. Plant Sci. 160: 265–272.

Rooney, L.W. and C.M. McDonough. (1987). Food quality and consumer acceptance in pearl millet, p. 43–61. In: J.R. Witcombe and S.R. Beckerman (eds.). Proc. International Pearl Millet Workshop. ICRISAT, Patancheru, India.

Roxas V.P., Sundus A.L., Garrett D.K., Mahan J.R. and Allen R.D. (2000). Stress tolerance in transgenic tobacco seedlings that over express glutathione S-transferase/glutathione peroxidase. Plant Cell Physiol. 41: 1229-1234.

Rozen S. and Skaletsky H.J. (2000). Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S and Misener S (eds), Bioinformatics Methods and Protocols: Methods in Molecular Biology, pp 365–386, Humana Press, Totowa, NJ.

Rudolph A.S., Crowe J.H. and Crowe L.M. (1986). Effects of three stabilizing agents proline, betaine and trehalose- on membrane phospholipids. Arch. Biochem. Biophys. 245: 134–143.

Ruegsegger A., Schmutz D., Brunold C. (1990). Regulation of glutathione synthesis by cadmium in *Pisum sativum* L. Plant Physiol. 93: 1579–1584.

Rus A., Yokoi S., Sharkhuu A., Reddy M., Lee B.H., Matsumoto T.K., Koiwa H., Zhu J.-K., Bressan R.A. and Hasegawa P.M. (2001). AtHKT1 is a salt tolerance determinant that controls Na^+ entry into plant roots. Proc. Natl. Acad. Sci. USA 98: 14150-14155.

Sadiqov S.T., Akbulut M. and Ehmedov V. (2002). Role of Ca^{2+} in drought stress signaling in wheat seedlings. Biochem. (Moscow) 67: 491–497.

Saghai Maroof M.A., Biyashev R.M., Yang G.P., Zhang Q and Allard R.W. (1994). Extraordinarily polymorphic microsatellite DNA in barley: Species diversity, chromosomal locations and population dynamics. Proc. Natl. Acad. Sci. USA 91: 5466–5470

Sairam R.K. and Tyagi A. (2004). Physiology and molecular biology of salinity stress tolerance in plants. Curr. Sci 86: 407–421.

Sairam R.K., Veerabhadra Rao K. and Srivastava G.C. (2002). Differential response of wheat genotypes to long term salinity stress in relation to oxidative stress, antioxidant activity and osmolyte concentration. Plant Sci. 163: 1037-1046.

Salin M.L. and Bridges S.M. (1980). Isolation and characterization of an iron-containing superoxide dismutase fron. a eukaryote, *Brassica campestris*.Arch. Biochem. Biophys. 201: 369–374.

Sangam S. (1995). Genetic and biochemical basis of salt tolerance in rice *Oryza sativa* L. at whole plant level. Ph.D. thesis. Department of Genetics, Osmania University, Hyderabad, A.P., India, pp.125.

Sangam S., Jayasree D., Janardhan Reddy K., Chary P.V.B., Sreenivasulu N. and Kavi Kishor P.B. (2005). Salt tolerance in plants – transgenic approaches. J. Plant Biotechnol. 7: 1–15.

Santos C.L.V., Campos A., Azevedo H and Caldeirs G. (2001). *In situ* and *in vitro* senescence induced by KCl stress: nutritional imbalance, lipid peroxidation and antioxidant metabolism. J. Exp. Bot. 52: 351-360.

Sarkar G., Cassady J., Bottema C.D.K. and Sommer S.S. (1990). Characterization of polymerase chain reaction amplification of specific alleles. Anal. Biochem. 186: 64-68.

Sarvesh A., Anuradha M., Pullaiah T., Reddy T.P. and Kavi Kishor P.B. (1996). Salt stress and antioxidant response in high and low proline producing cultivars of niger, *Guizotia abyssinica* (L.F.) Cass. Ind. J. Exp. Biol. 34: 252–256.

Sax K. (1923). The association of size differences with seed coat pattern and pigmentation in Phaseolus vulgaris. Genetics 8:552–60

Saxena O.P. and Pandey A.V. (2001). Hormonal modulation for crop productivity of low quality seeds. In: Khan NA, Samiullah (ed.), Phytohormones in Crop Productivity under Different Environments, Scientific Publishers, Jodhpur, India, pp. 135–166.

Sayed J. (1985). Diversity of salt tolerance in a germplasm collection of wheat (*Triticum aestivum*). Theor Appl. Genet. 69:651–657.

Scandalios J.G. (1993). Oxygen stress and superoxide dismutase. Plant Physiol. 101: 7–12.

Scandalios J.G. (1997). "Molecular Genetics of Superoxide Dismutases in Plants." In Oxidative Stress and the Molecular Biology of Antioxidative Defenses. (J.G. Scandalios, eds) pp 527–568. Plainview, Cold Spring Harbor.

Schaedle M. and J. Bassham (1977). Chloroplast glutathione reductase. Plant Physiol. 59: 1011-1012.

Semikhodskii A.G., Quarrie S.A. and Snape J.W. (1997). Mapping quantitative trait loci for salinity responses in wheat. *In* Drought and Plant Production. pp. 83-92. Proceedings of Lipenski Vir Meeting, Serbia 1996.

Senthilvel S., Mahalakshmi V., Sathish Kumar P., Reddy A.R., Markandeya G.,. Reddy M.K, Misra R. and Hash C.T. (2004). New SSR markers for pearl millet from data mining of Expressed Sequence Tags. Page 232 *in* Abstracts of the 4th International Crop Science Congress, Brisbane, Australia, 26 Sept. - 1 Oct. 2004.

Serna-Saldivar, S.O., C.M. McDonough, and L.W. Rooney. 1990. The millets, p. 271-300. In: K.J. Lorenz and K. Kulp (eds.). Handbook of cereal science technology. Markel Dekker, New York.

Serrano R., Culiañz-Maciá A. and Moreno V. (1999). Genetic engineering of salt and drought tolerance with yeast regulatory genes. Sci. Hortic. 78: 261-269.

Serrano R., Mulet J.M., Rios G., Marquez J.A., de Larrinoa I.F., Leube M.P., Mendizabal I., Pascual-Ahuir A., Proft M., Ros R. and Montesinos C. (1999). A glimpse of the mechanisms of ion homeostasis during salt stress.J. Exp. Bot. 50: 1023-1036.

Shalata A. and Tal M. (1998). The effects of salt stress on lipid peroxidation and antioxidants in the leaf of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii*. Physiol. Plant. 104: 169-174.

Shalata A., Mittova V., Volokita M., Guy M. and Tal M. (2001). Response of the cultivated tomato and its wild salt tolerant relative *Lycopersicon pennellii* to salt-dependant oxidative stress: the root antioxidative system. Physiol. Plant. 112: 487-494.

Shannon M.C. (1984). Breeding, selection and the genetics of salt tolerance. In: Staples, R.C. (Ed.), Salinity Tolerance in Plants: Strategies for Crop Improvement, pp. 231-254, Wiley, New York, NY.

Shannon M.C. (1985). Principles and strategies in breeding for salt tolerance. Plant & Soil 89: 227-241.

Shannon M.C., C. M. Grieve and L.E. Francois (1994). Whole-plant response to salinity. p. 199-244. In R.E. Wilkinson (ed.) Plant environment interactions. Marcel Dekker, Inc., New York.

Shen B., Hohmann S., Jensen R. G. and Bohnert H. J. (1999). Roles of sugar alcohols in osmotic stress adaptation. Replacement of glycerol by mannitol and sorbitol in yeast. Plant Physiol. 121: 45-52.

Shen, B., Jensen, R.G. and Bohnert, H. (1997). Increased resistance to oxidative stress in transgenic plants by targeting mannitol biosynthesis to chloroplasts. Plant Physiol. 113: 1177-1183.

Shinozaki K. and Yamaguchi-Shinozaki K. (1997) Gene expression and signal transduction in water-stress response. Plant Physiol. 115: 327-334.

Shirata K. and Takagishi H. (1990). Salt-induced accumulation of 26 and 27 kD proteins in cultured cells of rice plant. Soil Sci. Plant Nutr. 36: 153-157.

Sigma Plot (2001). Sigma Plot 2001 for Windows. Version 7.1, Copyright© 1986-2001, SPSS Inc.

Singh K.N. and Chatrath R. (2001). Salinity tolerance. *In* Reynolds, M.P., J.I. Ortiz-Monasterio, and A. McNab (eds.). Application of Physiology in Wheat Breeding. Mexico, D.F.: CIMMYT.

Singh K.N. and Rana R.S. (1989). Seedling emergence rating: A criterion for differentiating varietal responses to salt stress in cereals. Agric. Sci. Digest 9: 71-73.

Singh N.K., Braker C.A., Hasegawa P.M., Handa A.K., Buckels M.A., Hermodsm P., Franckoch E., Regnier F.E. and Bressan R.A. (1987a). Characterisation of osmotin athoumatin like protein associated with osmotic adaptation in plant cell. Plant Physiol. 85: 529-536.

Singh N.K., Handa A.K., Hasegawa P.M. and Bressan R.A. (1985). Proteins associated with adaptation of cultured tobacco cells to NaCl. Plant Physiol. 79: 126-137.

Singh N.K., Larosa P.C., Handa A.K., Hasegawa P.M. and Bressan R.A. (1987b). Hormonal regulation of protein synthesis associated with salt tolerance in plant cell. Proc. Natl. Acad. Sci. USA. 84: 739-743.

Singh S.D., Alagarswamy G., Talukdar B.S. and Hash C.T. (1994). Registration of ICML 22 photoperiod insensitive, downy mildew resistant pearl millet germplasm. Crop Sci. 34: 1421.

Singh S.D., Singh P., Rai K.N. and. Andrews D.J (1990). Registration of ICMA 841 and ICMB 841 pearl millet parental lines with A1 cytoplasmic-genic male-sterility system. Crop Sci. 30: 1378.

Singh S.D., Wilson J.P., Navi S.S., Talukdar B.S., Hess D.E. and Reddy K.N. (1997). Screening techniques and sources of resistance to downy mildew and rust in pearl millet. (In En. Summaries in En, Fr, Es.) Information Bulletin no. 48 ICRISAT, Patancheru 502324, India.

Sivakumar P., Sharmila P. and Pardha Saradhi P. (2000). Proline alleviates salt stress induced enhancement in ribulose 1,5-bisphosphate oxygenase activity. Biochem Biophys Res Commun. 279: 512-515.

Smirnoff N. (1998). Plant resistance to environmental stress. Curr. Opin. Biotech. 9: 214–219.

Smirnoff N. and Cumbes Q.J. (1989). Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28: 1057–1060.

Smith M.S., Yell Yang Y. and Yuji Kamiya (1995). Environmental controls on the early growth and development of the red mangrove, *Rhizophora mangle* L.: relationship to endogenous gibberellins. pp. 150–160. *In* M. Ajmal Khan & I.A. Ungar (eds.). Biology of Salt Tolerant Plants. University of Karachi, Karachi..

Smith R.L., Jensen L.S., Hoveland C.S. and Hanna W.W. (1989). Use of pearl millet, sorghum, and triticale grain in broiler diets. J. Prod. Agr. 2:78–82.

Sreenivasulu N., Grimm B., Wobus U. and Weschke W. (2000). Differential response of antioxidant compounds to salinity stress in salt-tolerant and salt-sensitive seedlings of foxtail millet (*Setaria italica* L.), Physiol. Plant. 109: 435–442.

Srivastava J.P. and Jana S. (1984). Screening wheat and barley germplasm for salt tolerance. *In* Salinity Tolerance in Plants: Strategies for Crop Improvement. Ed. RC Staples and GH Toenniessen. pp. 273-283. Wiley, New York, USA.

Stam P. (1993). Construction of integrated genetic linkage map by means of a new computer package: Joinmap. Plant J. 3: 739–744.

Staub J.E., Serquen F.C. and M. Gupta (1996). Genetic markers, map construction, and their application in plant breeding. Hort Sci. 31: 729–739.

Stegmeier W.D., Andrews D.J., Rai K.N. and Hash C.T. (1998). Pearl millet parental lines 843A and 843B. Int. Sorghum Millets Newsl. 39: 129–130.

Stuber C. (1995). Yield improvement success using marker facilitated QTL transfer between maize lines.
http://probe.nalusda.gov:8000/otherdocs/pg/pg3/14pg3.html

Stuber C.W. and Goodman M.M. (1983). Allozyme genotypes for popular and historically important inbred lines of corn. Southern series, No.16, United States Department of Agriculture, Agriculture Research Service.

Sudhakar C., Lakshmi A. and Giridarakumar S. (2001). Changes in the antioxidant enzyme efficacy in two high yielding genotypes of mulberry (*Morus alba* L.) under NaCl salinity. Plant Sci. 161: 613–619.

Sullivan, T.W., Douglas J.H., Andrews D.J , Bond P.L., Hancock J.D., Bramel-Cox P.J., Stegmeier W.D., and Brethour J.R. (1990). Nutritional value of pearl millet for food and feed. Proc. Int. Conf. in Sorghum Nutritional Quality, Purdue Univ., Lafayette, IN. pp. 83–94.

Sultana N., Ikeda T. and Kashem M.A. (2001). Effect of foliar spray of nutrient solutions on photosynthesis, dry matter accumulation and yield in seawater stressed rice. Environ. Exp. Bot. 46: 129–140.

Szabolcs I. (1992). Salinization of soils and water and its relation to desertification. Desertification Control Bulletin 21: 32–37.

Talukdar B.S., Prakash Babu P.P., Rao A.M., Ramakrishna C., Witcombe J.R., King S.B. and Hash C.T. (1998). Registration of ICMP 85410: Dwarf, downy mildew resistant, restorer parental line of pearl millet. Crop Sci. 38: 904–905.

Tanaka Y., Hibino T., Hayashi Y., Tanaka A., Kishitani S., Takabe T., Yokota S. and Takabe T. (1999). Salt tolerance of transgenic rice overexpressing yeast mitochondrial Mn-SOD in chloroplasts. Plant Sci. 148: 131–138.

Tanksley S.D. (1993). Mapping polygenes. Annu. Rev. Genet. 27: 205–234.

Tanksley S.D., Ganal M.W., Prince J.P., de Vincente M.C., Bonierbale M.W., Broun P., Fulton Y.M., Govannoni J.J., Grandillo S., Martin G.B., Messeguer R., Miller J.C., Miller L., Paterson A.H., Pineda O., Roder M.S., Wing R.A., Wu W. and Young N.D. (1992). High density molecular linkage maps of the tomato and potato genomes. Genetics 132: 1141–1160.

Tanksley S.D., Medina-Filho H. and Rick C.M. (1982). Use of naturally occurring enzyme variation to detect and map genes controlling quantitative traits in an interspecific backcross of tomato. Heredity 49: 11–25.

Tanksley S.D., Young N.D., Paterson A.H. and Bonierbale M. (1989). RFLP mapping in plant breeding: New tools for an old science. Biotechnology 7: 257–264.

Taramino G. and S. Tingsey (1996). Simple sequence repeats for germplasm analysis and mapping in maize. Genome 39: 277 287.

Tegelstrom H. (1992). Detection of mitochondrial DNA fragments in molecular genetic analysis of populations: A practical approach (Hoelze A R, ed), pp. 89–114, IRL Press, Oxford.

Thoday JM. (1961). Location of polygenes. Nature 191: 368–70.

Tsugane K., Kobayashi K., Niwa Y., Ohba Y. and Wada K.A. (1999). Recessive Arabidopsis mutant that grows photoautotrophically under salt stress shows enhanced active oxygen detoxification. Plant Cell 11: 1195–1206.

Ungar I.A. (1996). Effect of salinity on seed germination, growth and ion accumulation of *Atriplex patula* (*chenopodiaceae*). Am. J. Bot. 83: 604–607.

Utley H.G., Bernheim F. and Hochstein P. (1967). Effect of sulfhydryl reagents on peroxidation of microsomes. Arch. Biochem. Biophys. 118: 29-32.

Utz H. and A. Melchinger (1996). PLABQTL: A program for composite interval mapping of QTL. J Quant Trait Loci 2(1).

Utz H.F. and A.E. Melchinger (2000). PLABQTL: A computer program to map QTL (version 1.1). Institut für Pflanzenzüchtung, Saatgutforschung und Populationsgenetik, Universitaet Hohenheim, D-70593 Stuttgart, Germany.

Utz H.F. and Melchinger A.E. (2003). PLABQTL A computer program to map QTL, Version 1.2, 2nd edition.

Vaidyanathan H., Sivakumar P., Chakrabarty R. and George T. (2003). Scavenging of reactive oxygen species in NaCl-stressed rice (*Oryza sativa* L.)-differential response in salt-tolerant and sensitive varieties. Plant Sci. 165: 1411–1418.

Varshney K.A. and Baijal B.D. (1977). Effect of salt stress on seed germination of some pasture grasses. Comp. Physiol Ecol. 2: 104–107.

Vicente O., Boscaiu M., Naranjo M.A., Estrelles E., Belles J.M. and Soriano P. (2004). Responses to salt stress in the halophyte *Plantago crassifolia* (*Plantaginaceae*). J. Arid Environ. 58: 463–481.

*__Vinchon R. (1949).__ Étude sur la génétique des Pennisetum cultivés (Study of the genetics of the cultivated *Pennisetum* varieties). Agron Trop. 4: 451–485.

Virk P.S., Zhu J., Newbury H.J., Bryan G.J., Jackson M.T. and Ford-Lloyd B.V. (2000). Effectiveness of different classes of molecular markers for classifying and revealing variations in rice (*Oryza sativa*) germplasm. Euphytica 112: 275–284.

Vitoria A.P., Lea P.J. and Azevedo R.A. (2001). Antioxidant enzymes responses to cadmium in radish tissues. Phytochemistry 57: 701–710.

Vos P., Hogers R., Bleeker M., Reijans M., van de Lee T., Hoernes M., Frijters A., Pot J., Peleman J., Kuiper M. and Zabeau M. (1995). AFLP: A new technique for DNA fingerprinting. Nucleic Acids Res. 23: 4407–4414.

Wahome P.K., Jesch H.H. and Grittner I. (2001). Mechanisms of salt stress tolerance in two rose rootstocks :*Rosa chinensis* 'Major' ans *R.rubiginosa*. Sci. Hort. 87: 207–216.

Welsh J. & McClelland M. (1990). Fingerprinting genomes using PCR with arbitrary primers. Nucl. Acids Res. 18: 7213-7218.

Westman A.L. and Kresovich S. (1997). Use of molecular marker techniques for description of plant genetic variation. In: Biotechnology and Plant Genetic Resources: Conservation and Use. (Eds. Callow, J.A., J.A. Ford-Lloyd and H.J. Newbury). CAB International, Oxford, UK, pp. 9–48.

Williams J., Kubelik A., Livak K., Rafalski J. and Tingey S. (1990). DNA Polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res. 18: 6531–6535.

Williams M.N.V., Pande N., Nair S., Mohan M. and Bennet J. (1991). Restriction fragment length polymorphism analysis of polymerase chain reaction products amplified from mapped loci of rice (Oryza sativaL.) genomic DNA. Theor. Appl. Genet. 82: 489–498.

Williamson G. and Beverly M.C. (1987). The purification of acidic GSTs from pea seeds and their activity with lipid peroxidation products. Biochem. Soc. Trans. 15: 1103–1104.

Winter P. and Kahl G. (1995). Molecular marker technologies for plant improvement. World J. Microbiol. Biotech. 11: 438–448.

*__Wright S. (1934).__ An analysis of variability in number of digits in an inbred strain of guinea pigs. Genetics 19: 506–536.

www.ebi.ac.uk/clustalw/

www.gramene.org/multi/blastview

www.ncbi.nlm.nih.gov

www.ncbi.nlm.nih.gov/gorí/gorf.html

Wyn Jones R.G. and Pollard A. (1983). Proteins, enzymes and inorganic ions.*In* Encyclopedia of Plant Physiology. New Series, vol15.Eds. A.Lauchli and R.L. Bieleski. pp 528–562. Springer-Verlag, Berlin.

Xiang C. and Oliver D. (1998). Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in Arabidopsis. Plant Cell 10: 1539–1550.

Xu S.S., Hu J. and Faris J.D. (2003). Molecular characterization of Langdon durum-Triticum dicoccoides chromosome substitution lines using TRAP (target region amplification polymorphism) markers. In: Proc 10th Int Wheat Genet Symp, vol 1. Istituto Sperimentale per la Cerealicoltura, Rome, Italy, pp 91–94.

Yadav R.S., Bidinger F.R., Hash C.T., Yadav Y.P., Yadav O.P., Bhatnagar S.K. and Howarth C.J. (2003). Mapping and characterisation of QTL × E interactions for traits determining grain and stover yield in pearl millet. Theor. Appl. Genet. 106: 512–520.

Yadav R.S., Hash C.T., Bidinger F.R., Cavan G.P. and Howarth C.J. (2002). Quantitative trait loci associated with traits determining grain and stover yield in pearl millet under terminal drought-stress conditions. Theor. Appl. Genet. 104: 67–83.

Yadav R.S., Hash C.T., Bidinger F.R., Devos K.M. and Howarth C.J. (2004). Genomic regions associated with grain yield and aspects of post flowering drought tolerance in pearl millet across stress environments and tester background. Euphytica 136: 265–277.

Yang S.X., Zhao Y.X., Zhang Q., He Y.K., Zhang H. and Luo D. (2001). HAL 1 mediated salt adaptation in *Arabidopsis thaliana*. Cell and Research 11: 142–148.

Yao M.-Z. Wang J.-F. Chen H.-Y. Zhai H.-Q. and Zhang H.-S. (2005). Inheritance and QTL mapping of salt tolerance in rice. Rice Sci. 12: 25–32.

Yeo A. (1998). Molecular biology of salt tolerance in the context of whole–plant physiology. J.Exp.Bot. 49: 915–929.

Yeo A.R. (1983). Salinity resistance: Physiologies and prices. Physiol. Plant. 58: 214–222.

Yeo A.R. and Flowers T.J. (1986). Salinity resistance in rice (*Oryza sativa* L.) and a pyramiding approach to breeding varieties for saline soils. Aust. J. Plant Physiol. 13: 161–73.

Yeo A.R., Yeo M.E., Flowers S.A. and Flowers T.J. (1990). Screening of rice (*Oryza sativa* L.) genotypes for physiological characters contributing to salinity resistance, and their relationship to overall performance. Theor. Appl. Gen. 79: 377–384.

Yermiyahu U., Nir S., Ben-Hayyim G., Kafkafi U. and Kinraide T.B. (1997). Root elongation in saline solution related to calcium binding to root cell plasma membranes. Plant Soil 191: 67–76.

Young N.D. (1996). QTL mapping and quantitative disease resistance in plants. Annu. Rev. Phytopathol. 34: 479–501.

Young N.D. (2001). Constructing a plant genetic linkage map with DNA markers. In: DNA Based Markers in Plants. (Eds. Phillips, R.L. and I.K. Vasil). Kluwer Academic Publishers, The Netherlands. pp. 31–48.

Young N.D. and Tanksley S.D. (1989). restriction fragment length polymorphism maps and the concept of graphical genotypes. Theor. Appl. Genet. 77: 31–48.

***Zabeau M. and Vos P. (1993).** European Patent Application. publ. no. EP. 0534858

Zeitkiewiez E., Rafalski A. and Labuda D. (1994) Genomic fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20: 176–183.

Zeng Z.-B. (1993). Theoretical basis for separation of multiple linked gene effects in mapping quantitative trait loci. Proc. Natl. Acad. Sci. USA 90: 10972–10976.

Zeng Z.-B. (1994). Precision mapping of quantitative trait loci. Genetics 136: 1457–1468.

Zhang C-S., Lu Q. and Verma D.P.S. (1997). Characterization of δ^1-pyrroline-5-carboxylate synthetase gene promoter in transgenic *Arabidopsis thaliana* subjected to water stress. Plant Sci. 129: 81–89.

Zhang, G. Y., Guo, Y., Chen, S. L., and Chen, S. Y. (1995). RFLP tagging of a salt tolerance gene in rice. Plant Sci. 110: 227–234.

Zhu J.-K. (2000). Genetic analysis of plant salt tolerance using *Arabidopsis*. Plant Physiol. 124: 941–948.

Zhu J.K. (2001a). Plant salt tolerance. Trends Plant Sci 6: 66–71.

Zhu J.K. (2001b). Cell signaling under salt, water and cold stresses. Curr. Opin. Plant Biol. 4: 401–406.

Zhu J.K. (2002). Salt and drought stress signal transduction in plants. Annu. Rev. Plant Biol. 53: 247–273.

Zhu J.K. (2003). Regulation of ion homoeostasis under salt stress. Curr. Opin. Plant Biol. 6: 441–445.

Zhu J.K., Hasegawa P.M. and Bressan R.A. (1997). Molecular aspects of osmotic stress in plants. Crit. Rev. Plant Sci. 16: 253–277.

[* Originals not seen]

PUBLICATIONS

CHAPTER 8

PUBLICATIONS

Research paper(s) published:

1. **R. Mukhopadhyay, Hash C.T., Bhasker Raj A.G. and Kavi Kishor P.B. (2005).** Assessment of opportunities to map pearl millet tolerance to salinity during germination and early seedling growth. Internat Sorghum and Millets Newslett 46:117–120.

Research paper(s)/ poster(s) presented in Conferences/Symposia:

1. **R. Mukhopadhyay, C. T. Hash, A. G. Bhasker Raj, P. B. Kavi Kishor. (2005).** Assessing opportunities to map pearl millet tolerance to salinity during germination and early seedling growth. *at* '**International Conference on Sustainable Crop Production in Stress Environmants : Management and Genetic Options**'; February 9-12 2005; Jabalpur, Madhya Pradesh, India.

2. **R. Mukhopadhyay, V. Rajaram, P. Ramu, P.B. Kavi Kishor, S. Senthilvel, Jayashree B. and C.T. Hash. (2006)**. Target Region Amplification Polymorphism (TRAP): A novel approach for development of trait-specific markers in pearl millet for genes related to salinity stress tolerance. *at* '**International Symposium on Frontiers of Genetics and Biotechnology-Retrospect and Prospect**', Jan 8-10 2006, Osmania University, Hyderabad, Andhra Pradesh, India.

3. **R. Mukhopadhyay, M.S.L. Sunitha, S.P. Deshpande, P.B. Kavi Kishor, A.G. Bhaskar Raj, S. Senthilvel and C.T. Hash. (2006).** Target Region Amplification Polymorphism (TRAP): A new tool for mapping quantitative traits in pearl millet. *at* '**International Symposium on Frontiers of Genetics and Biotechnology-Retrospect and Prospect**', Jan 8-10 2006, Osmania University, Hyderabad, Andhra Pradesh, India.

4. **R. Mukhopadhyay, V Rajaram, P. Ramu, C. Ashok Kumar, P.B. Kavi Kishor, S. Sivaramakrishnan, S. Senthilvel, B. Jayashree and C. T. Hash. (2006).** Development of TRAP markers in pearl millet for genes related to nutritional quality of straw, terminal drought tolerance, and salinity stress tolerance. *at* '**Plant & Animal Genomes XIV Conference**', January 14-18, 2006, Town & Country Convention Center; San Diego, CA.

[For the posters 2 and 3, the research scholar won the **first prize** for poster presentation among 66 other equally competitive posters in that session].

APPENDICES

Appendix I

Primer	Query Sequence Accession No. (Maize)	Query Location: Details of the EST	Aligned sequences: Accession No. (Barley)	Accession No. (Millet)	Accession No. (Rice)	Accession No. (Sorghum)
GR6	*CN844501*	*EST2451 Zea mays embryo sac cDNA library Zea mays cDNA clone ES5882 5' similar to Glutathione reductase, mRNA sequence*	*BJ452669*	-	*AU102120*	*CD425598*
GR8	*CK787292*	*EST1931 Zea mays embryo sac cDNA library Zea mays cDNA clone ES4772 5' similar to Glutathione reductase, mRNA sequence*	*CA032471*	*CD725542*	*CB671323*	*CN129098*
GR13	*CF349127*	*EST06?3 Zea mays embryo sac cDNA library Zea mays cDNA clone ES1402 5' similar to Glutathione reductase, mRNA sequence*	*BU988162*	*CD725377*	*AU056093*	*BM325129*
SOD2	*CN844460*	*EST2410 Zea mays embryo sac cDNA library Zea mays cDNA clone ES5812 5' similar to Superoxide dismutase, mRNA sequence*	*BJ465242*	*CD724944*	*CF304545*	*AW678483*
SOD3	*CK849972*	*EST2014 Zea mays embryo sac cDNA library Zea mays cDNA clone ES5695 5' similar to Superoxide dismutase, mRNA sequence*	*BM374066*	*CD724944*	*AU184170*	*BF422046*
SOD5	*CK786884*	*EST1523 Zea mays embryo sac cDNA library Zea ma's cDNA clone ES 5' similar to Superoxide dismutase, mRNA sequence*	*AV913785*	*CD724944*	*CF321285*	*BG241127*
SOD6	*CK786803*	*EST1442 Zea mays embryo sac cDNA library Zea mays cDNA clone ES3371 5' similar to Superoxide dismutase, mRNA sequence*	*AV913785*	*CD724944*	*BAA00800*	*BE917823*
SOD7	*BQ619487*	*RNOSEQ8C04_SK.ab1 Salt stressed Zea mays roots cDNA library Zea mays cDNA clone RNOSEQ8C04_SK.ab1 similar to superoxide dismutase (EC 1.15.1.1) (Mn) sod3 precursor [validated] - maize gi|168624|gb AAA33512.1| (M33119) manganese superoxide dismutase (SOD-3) (EC 1.15.1.1) [Zea mays], mRNA sequence*	*AJ462195*	*CD725382*	*AU182793*	*AI724467*
P5CS	*CN845408*	*EST3359 Zea mays embryo sac cDNA library Zea mays cDNA clone ES7657 5' similar to Delta 1-pyrroline-5-carboxylate synthetase, mRNA sequence*	*CA024010*	-	*CA754084*	*BM329987*

Appendix II
Composition of Solutions and Reagents

Composition of Hoagland solution (Hoagland & Arnon, 1938)

A. Macronutrients	**g/l**
$Ca(NO_3)_2\ 4H_2O$	0.94
$MgSO_4\ 7H_2O$	0.52
KNO_3	0.66
$NH_4\ H_2PO_4$	0.12
Iron chelate sequestrate(/$FeSO_4$)	0.07
B. Micronutrients	**g/l**
H_3BO_3	28.0
$MnSO_4\ H_2O$	34.0
$CuSO_4\ 5H_2O$	1.0
$ZnSO_4\ 7H_2O$	2.2
$(NH_4)_6MO_7\ 4H_2O$	1.0
H_2SO_4 (conc.)	5 ml

Add 0.1 ml of micronutrients after making up the macronutrients solution to 1 liter with distilled water. Adjust pH of the solution to 6.7 at 20°C.

CTAB (Cetyl Trimethyl Ammonium Bromide) (2 %) buffer

CTAB	20 g
1 M Tris	200 ml
5 M NaCl	280 ml
0.5 M EDTA	40 ml
Na2SO3	2.5 g
Distilled water	460 ml

Add mercaptoethanol (0.1 %) fresh while using CTAB (2 %) solution.

Rnase (10 mg/ ml)

Dissolve Rnase in water, place in a tube in a boiling water bath for 10 minutes. Allow this to cool on a bench and store at -20 °C.

Chloroform: isoamyl alcohol (24:1)

Chloroform	240 ml
Isoamyl alcohol	10 ml

Store in dark at room temperature. Make up and dispenses the solution in a fumed cupboard.

Ethanol (70 %)

Absolute alcohol	70 ml
Distilled water	30 ml

NaCl (5 M)

Dissolve 292.2 g NaCl in 750 ml water. Make up to 1 liter with water, filter and autoclave.

Phenol/ Chloroform

Mix equal volume of the buffered phenol and chloroform: isoamyl alcohol (24:1). Store at 4°C.

Sodium acetate (2.5 M, pH 5.2)

Dissolve 340.2 g sodium acetate in 500 ml water. Adjust pH to 5.2 with glacial acetic acid and make volume up to 1 liter and autoclave.

Tris HCl (1M, pH 8.0)

Dissolve 121.1 g Tris in 800 ml of water. Adjust pH to 8.0 with conc. HCl make volume up to 1 liter and autoclave.

EDTA (0.5 m, Ph 8.0)

Dissolve 186.1 g Na_2 EDTA.$2H_2O$ in 800 ml water. Adjust pH to 8.0 with Sodium hydroxide pellets. Make up volume to 1 liter and autoclave.

$T_{10}E_1$ buffer

1M Tris HCl pH 8.0	10 ml
1M EDTA pH 8.0	1 ml

And make up to 1 liter with sterile distilled water.

$T_{50}E_{10}$ buffer

1M Tris HCl pH 8.0	50 ml
0.5 M EDTA pH 8.0	20 ml

Make volume up to 1 liter with sterile distilled water.

10X Tris-Borate Buffer (TBE) (per liter)

Tris buffer

Boric Acid

EDTA

108 g Tris base, 55 g Boric acid and 9.3 g EDTA. Add deionised H_2O to 1 liter. The pH is 8.3 and requires no adjustment.

6X Gel loading buffer (0.25 % Bromophenol blue, 40 % sucrose)(10 ml)

Sucrose	4 g
Bromophenol Blue	2.5 ml
dH_2O	up to 10 ml

Store at 4 °C.

Ethidium bromide (10 mg/ml)

Dissolve 100 mg ethidium bromide in 10 ml of distilled water; wrap tube in aluminium foil and store at 4°c.

Caution: Ethidium bromide is extremely mutagenic.

Acrylamide / biacrylamide 29:1 (w/w)

Acrylamide	29 g
Biacrylamide	1 g

Water (deionised distilled) up to 100 ml

Store at 4 °C for <= 1 month.

Acrylamide/bisacrylamide 29:1 (v/v)

Acrylamide	87 ml
Bisacrylamide	3 ml

Add deionised distilled water to 300 ml. Solution can be stored up to 1 month at 4°C.

10 % (W/V) Ammonium Per Sulphate

Ammonium per Sulphate 1 g

Water (deionised distilled) 10 ml

Make fresh stock every week and store at 4°C.

TEMED (N, N, N', N'-tetramethylethylenediamine)

Ready made, store between 10 and 30°C (check label flask).

Loading buffer for non-denaturing PAGE (5X)

50 mM EDTA (1 ml of 0.5 M EDTA, pH 8.0)

50 mM NaCl (100 µl of 5 M NaCl)

50% (v/v) glycerol (5 ml)

Make up to 9 ml with sterilized deionised water. Add 10 mg fast orange G dye and adjust the volume to 10 ml. If you are using bromophenol blue and cyanol then less is required.

Binding silane

0.15 ml Bind silane

0.5 ml Acetic Acid

99.35 ml Ethanol

Mix the ingredients and store at 4°C.

100 base pairs ladder (50ng/ml)

100 bp ladder (stock conc.1 µg/µl)	50 µl
Blue (6X dye)	165 µl
$T_{10}E_1$ buffer	785 µl

Repel silane

Ready made, store at 4°C.

Reagents used for the Silver staining for PAGE :

0.1 % (w/v) CTAB

2 gram CTAB in 2 liters of distilled deionised water

1 M NaOH (freshly prepared)

0.3 % liquid Ammonia

Wear face mask when handling ammonia, should preferably be done in fume cupboard

Silver nitrate solution (freshly prepared)

2 gram silver nitrate

8 ml 1M NaOH

6-8 ml 25% ammonia.

Dissolve the silver nitrate and NaOH into 2 liters of distilled deionised water. Titrate with ammonia (on a shaker) until the solution becomes clear; add a further 1 ml of ammonia solution.

Sodium Carbonate solution (freshly prepared)

Note that the Sodium Carbonate should not be older than 12 months)

Dissolve 30 g sodium carbonate in 2 liters of distilled deionised water. Add 0.4 ml formaldehyde.

Glycerol solution

30 ml Glycerol into 2 liters distilled deionised water.

Concentrated NaOH solution

40 gram into 1 liter of water.

Appendix III

Score-sheet for TRAP markers (Cross: Tift $23D_2B_1$-P1-P5 × WSIL-P8; $F_{2:4}$ Genotypes = 97)

S No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
$F_{2:4}$No	7	11	14	15	16	19	20	21	24	25	26	27	28	29	30	31	32	33	35	36	37	38	39	41	43	45	47
G6A2.260	C	C	C	A	A	C	C	C	C	A	A	A	C	A	C	A	A	C	C	C	C	C	A	C	C	A	C
G6A2.290	C	C	A	A	A	A	C	A	C	C	A	A	A	C	A	C	A	C	A	A	C	A	A	C	A	A	C
G6A2.350	C	A	C	A	A	C	A	C	A	A	C	A	C	A	C	A	A	C	A	C	A	C	A	C	A	A	A
G6A2.380	C	C	C	C	C	C	C	C	A	A	A	C	A	C	C	C	A	C	A	C	C	C	C	C	A	A	C
G6A2.450	B	B	B	B	B	B	D	B	B	B	B	B	D	B	D	D	B	B	D	D	D	D	B	B	B	B	B
G6A2.520	B	B	D	B	D	D	B	D	B	B	B	B	D	B	D	D	D	B	D	B	B	D	B	D	D	B	B
G6A2.560	B	D	B	D	B	D	D	D	B	D	D	D	D	D	B	B	D	D	D	D	B	D	D	D	D	D	D
G6A2.610	D	D	D	D	D	D	D	B	D	B	D	D	D	D	D	D	D	D	D	B	D	D	D	B	B	B	B
G6A2.870	A	A	C	A	A	C	A	A	A	A	A	A	A	A	C	A	A	A	A	A	A	A	A	C	A	A	A
G6A2.880	C	A	C	A	A	A	A	A	C	A	A	A	A	A	C	C	A	C	A	C	C	C	A	C	A	A	A
G8A3.1050	B	B	B	D	D	B	-	B	D	B	D	-	D	B	B	D	D	B	D	D	B	D	B	D	B	D	B
G8A3.195	C	C	C	A	C	C	A	A	C	A	A	C	C	C	C	A	C	C	C	C	C	C	C	C	C	C	C
G8A3.240	A	A	C	A	A	-	C	C	-	C	A	C	C	A	C	A	C	C	A	C	C	C	A	-	A	C	C
G8A3.360	D	D	D	D	D	B	B	D	D	B	D	B	D	D	B	D	D	D	B	B	D	D	B	D	D	D	D
G8A3.450	A	A	C	A	-	A	A	A	A	C	A	A	A	A	C	A	A	C	A	C	A	A	A	C	C	A	A
G8A3.480	D	D	D	D	D	B	B	D	D	D	D	B	B	D	B	D	B	B	D	D	D	B	D	B	B	D	D
G8A3.490	D	B	D	D	D	D	D	D	D	D	B	D	B	D	D	D	D	D	D	B	B	B	B	B	D	D	D
G8A4.240	C	A	C	C	C	A	A	C	C	A	A	C	A	A	C	C	C	A	A	C	C	A	C	A	C	A	C
G8A4.580	C	A	C	C	C	A	C	C	C	C	A	C	C	C	C	C	C	A	C	C	C	C	A	C	C	C	C
G8A4.680	C	C	A	A	A	C	A	C	C	A	A	A	C	C	A	C	C	C	C	C	C	C	C	A	A	A	A
G8A4.740	B	B	D	D	B	D	D	D	D	D	B	D	D	D	D	D	D	D	D	B	D	B	D	B	D	D	D
PA1.1040	A	C	A	C	C	A	C	A	C	C	C	C	C	C	A	C	C	C	A	C	C	A	C	A	C	C	C
PA1.220	D	D	D	B	D	D	D	D	D	D	D	D	B	D	D	D	D	D	D	D	D	D	D	B	D	B	D
PA1.380	D	B	D	D	D	D	B	D	B	B	D	D	B	B	B	D	D	D	D	D	B	D	B	D	B	D	D
PA1.520	C	C	C	A	A	C	C	C	A	-	C	C	-	C	C	A	C	C	C	C	C	C	A	C	A	C	C
PA1.550	B	D	D	B	D	D	B	D	D	D	B	D	B	D	D	D	D	D	D	D	D	D	D	D	D	D	D
PA1.590	C	C	C	A	C	C	C	A	C	A	C	C	A	C	A	A	A	C	A	C	C	C	A	C	A	C	C

Score-sheet for TRAP markers (Cross: Tift $23D_2B_1$-P1-P5 × WSIL-P8; $F_{2:4}$ Genotypes = 97) contd.

S.No.	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54
$F_{2:4}$No.	48	49	51	53	55	56	62	63	64	69	70	72	73	74	75	76	77	78	79	80	81	82	84	85	86	87	88
G6A2.260	C	A	A	A	A	A	C	C	A	C	C	A	A	A	A	A	C	A	C	A	C	A	C	A	C	A	A
G6A2.290	C	A	C	C	C	C	A	A	C	C	C	C	C	A	A	A	A	C	C	A	A	C	C	C	A	A	C
G6A2.350	C	A	A	C	A	A	A	C	A	A	A	C	A	A	A	A	A	A	A	A	C	A	A	A	A	C	C
G6A2.380	C	C	C	C	C	C	A	C	C	A	C	C	C	A	C	C	C	C	C	C	C	C	A	C	C	C	C
G6A2.450	B	B	B	B	D	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	D	B	B	B	B	B	B
G6A2.520	B	B	D	B	B	B	B	D	D	B	B	B	D	D	B	B	B	B	B	D	B	B	B	D	B	B	B
G6A2.560	B	B	B	D	B	D	D	D	D	D	D	D	B	D	D	D	D	D	D	D	D	D	D	D	D	D	D
G6A2.610	D	D	D	B	D	D	D	D	D	B	B	B	D	D	B	D	D	D	D	D	D	D	D	B	B	D	B
G6A2.870	A	C	A	C	A	A	A	A	A	A	C	C	C	A	A	C	A	A	A	A	C	A	C	A	A	A	C
G6A2.880	C	A	A	C	C	A	C	A	C	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
G8A3.1050	D	D	D	B	B	D	D	D	D	D	D	B	D	B	D	B	D	D	D	D	B	D	D	B	D	B	B
G8A3.195	C	A	C	C	A	A	C	C	C	C	C	C	C	C	C	C	C	A	C	A	C	A	A	C	A	C	C
G8A3.240	C	A	C	C	-	A	C	A	A	C	C	A	A	A	C	A	A	A	C	A	C	A	A	A	A	C	C
G8A3.360	D	D	D	D	B	D	B	B	B	B	B	D	B	B	D	D	D	D	D	D	D	B	D	D	B	B	D
G8A3.450	C	A	C	A	A	C	A	A	A	C	C	A	A	A	C	A	C	A	A	A	C	A	C	A	C	C	A
G8A3.480	B	D	D	B	B	D	D	B	B	B	D	B	B	D	B	B	D	D	D	D	B	D	D	D	D	D	B
G8A3.490	D	B	D	B	D	D	B	D	-	D	D	B	D	D	D	D	D	B	D	B	D	D	D	B	D	B	D
G8A4.240	C	C	C	A	C	A	C	C	A	A	A	C	C	C	C	A	C	C	C	C	C	A	C	A	C	C	C
G8A4.580	C	C	C	A	C	A	C	C	A	C	C	A	C	C	C	C	C	A	A	C	C	A	C	A	C	C	C
G8A4.680	C	C	C	C	A	C	C	C	C	A	A	C	C	A	A	C	A	C	A	A	C	C	A	C	C	C	A
G8A4.740	D	D	D	B	D	D	D	D	D	D	D	B	B	D	D	B	D	D	D	D	B	D	D	B	D	B	D
PA1.1040	C	A	C	A	C	C	C	C	C	C	C	C	A	A	C	C	C	C	C	A	C	C	C	C	C	C	C
PA1.220	D	D	B	D	B	D	D	B	D	D	B	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	B
PA1.380	B	D	D	D	D	B	D	D	D	D	D	B	D	D	B	D	D	B	B	D	D	D	B	D	D	D	B
PA1.520	C	C	-	C	A	C	C	C	C	C	C	C	C	A	C	C	C	A	C	A	C	A	C	C	C	C	C
PA1.550	D	D	D	D	D	D	B	D	D	D	D	B	B	D	D	B	D	B	D	D	D	D	D	D	D	D	D
PA1.590	A	C	A	C	A	C	C	C	C	C	C	C	C	C	C	C	A	C	A	A	C	C	A	C	C	C	A

Score-sheet for TRAP markers (Cross: Tift $23D_2B_1$-P1-P5 × WSIL-P8; $F_{2:4}$ Genotypes = 97) contd.

S.No.	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76
$F_{2:4}$No.	89	92	95	96	97	99	100	101	102	103	106	107	108	110	111	114	115	116	117	118	119	120
G6A2.260	A	A	A	C	C	A	C	C	C	C	A	C	-	A	C	C	C	C	A	A	A	C
G6A2.290	A	C	A	A	A	C	C	A	C	A	C	A	A	C	C	A	A	A	C	A	C	A
G6A2.350	A	C	C	A	C	A	A	A	C	C	C	A	A	A	A	C	C	A	A	A	A	A
G6A2.380	C	C	C	C	A	C	C	C	C	C	A	A	A	A	C	C	C	C	A	A	C	C
G6A2.450	B	B	B	B	D	B	B	B	B	D	B	D	B	B	B	B	D	D	B	B	B	B
G6A2.520	B	D	B	B	D	B	D	B	B	B	D	D	D	B	B	B	B	D	B	D	B	D
G6A2.560	D	D	B	-	D	B	D	D	D	D	D	D	D	D	D	D	D	D	D	D	B	D
G6A2.610	D	D	B	B	B	D	D	D	B	D	B	B	D	D	B	D	D	D	B	D	D	D
G6A2.870	A	C	C	A	A	A	A	A	A	A	A	A	A	A	A	C	C	C	A	A	A	A
G6A2.880	A	A	A	A	A	A	A	C	A	A	A	A	A	A	C	A	A	A	A	C	A	A
G8A3.1050	-	B	D	D	B	D	D	D	B	-	B	B	D	D	B	D	-	D	B	B	D	B
G8A3.195	A	C	C	C	C	C	C	C	C	A	C	C	C	-	C	C	C	C	A	C	A	C
G8A3.240	A	C	A	A	A	C	A	C	A	C	C	C	A	A	C	C	-	C	C	A	C	A
G8A3.360	B	D	D	B	D	D	B	D	D	D	D	B	D	D	B	D	B	D	D	D	D	D
G8A3.450	C	A	C	C	C	C	A	A	A	C	C	-	C	C	C	A	A	A	A	C	C	C
G8A3.480	B	D	B	B	D	B	D	D	B	D	B	B	B	D	D	D	B	D	B	D	D	D
G8A3.490	D	B	B	B	D	D	B	B	D	B	B	D	D	B	B	B	D	B	D	D	B	B
G8A4.240	A	A	C	C	A	C	C	C	C	C	A	C	C	C	C	C	C	C	C	A	C	C
G8A4.580	A	C	C	C	C	C	C	C	C	C	A	C	C	C	A	C	C	A	C	A	C	C
G8A4.680	A	C	C	C	A	C	C	C	C	C	A	A	A	A	C	A	C	C	A	A	C	A
G8A4.740	D	B	B	B	D	D	D	B	D	B	D	D	D	D	B	D	D	D	D	D	B	D
PA1.1040	A	C	A	C	A	C	C	C	C	C	C	A	A	C	C	C	C	A	C	C	C	C
PA1.220	D	D	D	D	D	D	D	D	D	D	B	D	D	D	D	D	D	D	D	D	B	D
PA1.380	D	D	D	D	D	D	D	D	D	D	B	D	D	B	B	D	D	D	B	D	D	D
PA1.520	C	C	A	C	C	C	C	C	C	C	A	C	A	A	C	C	C	A	C	C	C	C
PA1.550	D	D	D	D	D	D	D	D	D	D	D	D	D	D	B	D	D	B	D	D	D	D
PA1.590	C	C	C	C	A	A	C	C	C	C	A	A	C	A	C	C	C	A	C	C	C	C

Score-sheet for TRAP markers (Cross: Tift $23D_2B_1$-P1-P5 × WSIL-P8; $F_{2:4}$ Genotypes = 97) contd.

S.No.	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97
$F_{2:4}$No.	122	123	124	125	127	130	131	132	133	135	136	137	138	139	140	145	147	148	151	153	159
G6A2.260	A	A	C	C	A	C	A	C	A	A	C	A	C	A	C	A	C	C	C	C	A
G6A2.290	C	A	C	C	C	A	A	C	C	A	C	A	C	C	A	C	C	C	C	A	A
G6A2.350	A	A	A	A	A	A	C	C	C	A	A	A	A	C	C	A	C	C	A	C	C
G6A2.380	C	C	C	C	A	A	A	C	A	A	C	C	A	C	C	C	A	C	A	A	A
G6A2.450	B	B	B	B	B	B	B	B	D	B	B	D	B	B	B	D	B	B	B	-	-
G6A2.520	B	B	B	B	B	B	B	B	B	B	D	B	B	B	D	B	D	B	D	B	D
G6A2.560	D	D	D	D	D	D	D	D	-	B	D	D	D	D	D	D	D	D	D	D	D
G6A2.610	B	B	D	D	D	B	B	B	B	B	B	D	D	D	D	D	B	D	B	B	B
G6A2.870	C	C	C	C	A	A	A	C	A	A	C	C	A	C	C	C	A	C	A	C	C
G6A2.880	A	A	A	A	A	A	C	A	A	C	A	A	A	A	C	A	C	A	C	A	C
G8A3.1050	B	D	-	B	B	B	D	B	B	B	D	D	B	B	D	B	B	D	D	D	D
G8A3.195	C	C	C	C	C	C	C	C	C	C	C	C	C	C	A	A	C	A	C	A	A
G8A3.240	A	C	C	C	C	C	-	C	A	C	C	A	A	A	A	C	A	C	C	A	A
G8A3.360	D	D	D	D	B	D	D	B	D	B	B	D	D	D	D	B	D	D	B	D	D
G8A3.450	A	C	A	C	A	C	C	A	A	A	A	C	A	A	A	C	A	C	C	A	C
G8A3.480	D	B	B	B	B	D	D	B	B	D	B	D	B	D	D	D	D	B	D	B	B
G8A3.490	B	D	D	D	D	D	D	D	D	B	D	B	D	B	B	B	B	B	D	D	D
G8A4.240	C	A	A	C	A	A	A	C	A	C	A	A	C	A	C	A	A	A	A	A	A
G8A4.580	C	A	C	C	C	A	C	C	C	A	C	A	C	C	A	A	A	C	A	A	A
G8A4.680	A	C	C	A	A	A	C	A	A	A	C	C	A	C	C	C	A	C	A	A	A
G8A4.740	D	D	D	D	D	D	D	D	B	B	D	B	D	B	B	D	B	B	D	D	D
PA1.1040	C	C	C	C	C	C	C	C	A	A	C	C	C	C	C	C	A	C	C	C	A
PA1.220	B	D	D	D	D	D	B	D	D	B	D	D	D	D	D	B	D	D	B	B	B
PA1.380	D	D	D	B	B	D	D	B	B	B	D	D	B	B	D	D	B	B	B	B	B
PA1.520	C	C	C	C	C	C	A	C	A	A	C	C	C	C	C	A	A	C	-	-	-
PA1.550	D	B	D	D	D	D	D	D	D	D	D	D	B	D	D	D	D	B	D	D	B
PA1.590	A	C	C	C	C	A	C	A	A	A	C	C	C	C	C	A	C	C	A	A	A

Score-sheet for TRAP markers (Cross: Tift $23D_2B_1$-P1-P5 × WSIL-P8; $F_{2:4}$ Genotypes = 97) contd.

S.No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
$F_{2:4}$No.	7	11	14	15	16	19	20	21	24	25	26	27	28	29	30	31	32	33	35	36	37	38	39	41	43	45	47
PA1.805	D	D	D	B	D	D	D	D	D	B	D	D	D	B	D	D	D	D	B	D	D	D	D	D	D	D	B
PA1.910	C	A	C	C	C	A	C	A	C	C	A	C	C	C	C	C	C	A	C	C	C	C	C	C	C	C	C
PA1.990	C	A	C	C	C	A	C	A	C	C	A	C	C	C	C	C	C	C	A	C	C	C	A	C	A	A	C
PA15.1050	C	A	C	C	C	A	C	C	A	C	C	C	A	C	C	A	-	A	C	A	A	C	C	A	A	A	A
PA15.165	B	B	B	B	B	B	B	B	A	A	A	B	B	A	A	B	B	B	H	B	B	B	A	B	B	B	B
PA15.200	B	D	D	B	D	B	D	D	D	D	D	B	D	B	B	D	D	D	B	D	D	D	D	B	B	B	D
PA15.290	A	B	A	A	A	A	A	H	A	A	A	A	A	A	B	H	H	B	A	A	B	A	A	B	A	A	A
PA15.550	B	D	B	D	D	B	B	B	B	B	D	B	B	B	D	B	B	B	D	B	B	B	D	B	D	B	B
PA8.1050	A	A	A	C	A	C	A	C	A	A	A	C	C	C	A	C	A	A	A	C	A	C	C	C	A	A	A
PA8.130	D	B	D	D	B	D	B	D	B	B	D	D	B	B	D	B	B	D	D	D	B	D	B	D	B	D	B
PA8.510	C	C	A	A	A	C	C	C	A	A	A	C	A	C	C	A	C	C	C	A	A	C	C	C	A	A	C
PA8.990	A	C	A	A	C	A	A	C	A	C	A	A	C	C	C	C	A	A	A	A	A	C	A	C	A	A	A
S2A7.120	A	C	A	A	C	A	C	A	C	C	C	C	A	C	A	C	C	C	A	A	C	A	C	C	C	A	C
S2A7.1500	D	D	D	D	D	D	D	B	D	D	D	D	B	D	D	B	D	B	D	D	D	D	D	D	B	D	D
S2A7.630	A	A	A	H	A	H	H	A	H	A	A	A	H	H	A	H	B	A	H	A	A	A	A	H	A	H	H
S2A7.690	D	B	D	D	D	D	D	D	D	D	D	D	B	D	D	D	D	D	D	D	D	D	B	D	B	D	D
S2A7.880	D	B	D	B	D	B	B	B	D	B	B	D	B	B	D	D	D	B	B	D	D	D	B	D	D	D	D
S2A7.980	A	C	C	C	A	A	C	A	C	C	C	C	A	C	C	A	A	A	A	C	C	C	C	C	C	C	C
S3A11.1590	D	B	D	D	D	D	B	D	D	D	D	B	D	D	B	D	D	B	D	D	D	B	D	B	D	D	D
S3A11.450	A	A	A	C	A	C	C	C	A	A	A	A	A	C	A	A	A	A	A	A	A	A	A	C	A	C	C
S3A11.490	B	B	D	D	D	D	D	D	D	D	B	D	D	D	D	D	D	D	D	D	D	B	D	D	D	D	D
S3A11.760	A	C	A	A	A	C	C	C	A	A	A	C	C	C	C	C	A	C	A	A	A	C	A	A	A	C	C
S3A11.850	C	C	A	A	A	C	A	A	C	C	C	A	A	C	A	C	C	C	A	C	C	A	C	C	C	C	C
S3A4.1550	D	B	D	D	D	D	D	D	D	D	D	B	D	D	B	D	D	B	D	B	D	B	D	B	D	D	D
S3A4.530	B	A	B	H	B	B	H	-	H	-	B	A	-	H	B	B	B	B	B	B	-	H	H	B	B	B	-
S3A4.660	D	B	D	D	D	B	D	B	B	D	B	B	D	B	B	B	B	B	D	D	D	-	D	B	B	B	B
S3A4.890	A	C	A	C	C	C	C	A	C	C	C	C	A	C	A	C	C	C	A	A	C	A	C	C	C	C	C

Score-sheet for TRAP markers (Cross: Tift $23D_2B_1$-P1-P5 × WSIL-P8; $F_{2:4}$ Genotypes = 97) contd.

S.No.	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54
$F_{2:4}$No.	48	49	51	53	55	56	62	63	64	69	70	72	73	74	75	76	77	78	79	80	81	82	84	85	86	87	88
PA1.805	D	B	B	D	D	D	B	D	D	D	B	D	D	D	B	D	D	D	B	B	D	B	B	B	B	D	D
PA1.910	C	A	C	A	C	A	A	A	A	C	C	C	C	C	C	A	C	C	A	C	C	A	C	A	C	C	C
PA1.990	C	C	C	A	C	A	A	A	C	C	A	A	C	A	C	A	A	C	A	C	C	A	A	A	C	C	C
PA15.1050	A	A	A	A	C	A	A	C	A	A	A	C	A	A	A	A	A	C	C	C	C	A	C	A	A	A	C
PA15.165	A	B	B	B	B	A	B	A	B	B	B	B	A	B	A	B	B	B	B	B	B	A	A	B	B	B	B
PA15.200	B	D	D	B	B	D	D	B	D	D	D	D	D	D	B	B	D	D	D	D	B	B	B	B	D	B	D
PA15.290	A	A	A	A	A	A	A	A	A	A	A	A	H	A	A	A	A	A	H	A	H	A	A	A	A	A	B
PA15.550	B	D	B	B	B	D	B	D	B	B	B	B	D	D	B	B	D	D	D	D	B	B	D	B	B	B	B
PA8.1050	A	A	A	A	A	A	C	A	A	C	C	A	C	A	C	A	A	C	A	A	C	A	A	C	A	A	A
PA8.130	D	B	D	D	D	B	D	D	B	B	B	B	D	D	B	D	B	B	B	B	B	B	B	B	B	D	B
PA8.510	C	A	A	C	C	C	C	C	C	C	C	C	C	A	C	C	A	A	A	A	C	C	A	C	A	A	C
PA8.990	C	C	C	A	A	A	A	A	A	C	C	A	C	C	C	C	A	C	A	A	C	A	C	A	C	A	A
S2A7.120	C	A	A	A	C	C	A	A	C	C	C	A	C	A	C	C	C	C	C	A	C	C	C	C	C	C	C
S2A7.1500	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	B	D	D	D	D	D
S2A7.630	A	A	H	H	H	A	H	A	H	H	H	H	A	H	H	A	A	A	H	B	B	H	A	A	H	H	B
S2A7.690	D	B	D	D	D	D	D	D	D	D	D	D	D	D	B	D	D	D	D	D	B	D	D	B	D	D	D
S2A7.880	D	B	D	B	D	B	B	B	B	B	D	B	D	D	D	D	D	D	B	B	B	B	D	B	D	B	B
S2A7.980	C	A	A	C	C	C	C	C	A	C	C	C	C	C	A	C	A	C	A	C	C	C	A	C	A	C	C
S3A11.1590	B	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
S3A11.450	A	A	A	A	C	A	C	A	C	A	C	A	A	C	A	A	A	A	A	C	A	C	A	A	C	A	C
S3A11.490	D	B	B	B	D	D	D	D	D	B	D	B	D	D	D	B	D	D	D	D	B	D	D	D	D	D	D
S3A11.760	C	A	A	A	C	C	C	A	C	C	A	C	A	A	A	A	A	A	A	A	A	C	A	C	C	A	A
S3A11.850	A	C	C	C	C	C	C	A	C	C	C	C	C	A	C	C	C	C	C	C	C	C	C	C	C	A	C
S3A4.1550	D	D	D	-	D	D	D	D	B	D	B	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
S3A4.530	-	B	B	H	H	B	B	B	H	B	H	H	H	B	-	B	B	H	B	H	H	B	B	B	B	B	H
S3A4.660	D	B	B	B	B	D	B	D	B	D	D	B	B	B	D	B	B	B	B	B	D	B	B	B	B	B	B
S3A4.890	C	C	C	C	C	C	C	A	A	C	C	C	C	A	C	C	C	C	C	C	C	C	C	C	C	A	C

Score-sheet for TRAP markers (Cross: Tift $23D_2B_1$-P1-P5 × WSIL-P8; $F_{2:4}$ Genotypes = 97) contd.

S.No	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76
$F_{2:4}$No.	89	92	95	96	97	99	100	101	102	103	106	107	108	110	111	114	115	116	117	118	119	120
PA1.805	D	D	B	B	B	D	D	B	D	D	B	D	D	D	D	D	D	D	B	D	B	D
PA1.910	A	C	C	C	A	C	C	C	C	C	A	C	C	C	A	C	C	A	C	A	C	C
PA1.990	A	C	A	A	A	C	C	C	C	C	A	C	C	C	A	C	C	C	C	A	C	C
PA15.1050	A	A	-	-	C	C	C	C	C	C	A	C	C	-	C	A	C	C	C	A	A	C
PA15.165	B	B	-	B	B	B	B	B	B	B	A	B	H	-	B	B	B	B	B	B	A	B
PA15.200	D	D	-	B	D	D	D	D	D	D	B	D	D	-	B	D	D	D	B	D	B	D
PA15.290	H	A	-	A	A	A	A	A	A	A	B	A	A	-	B	A	A	A	B	A	A	A
PA15.550	D	D	-	D	B	B	B	B	D	B	B	B	D	-	B	B	D	B	B	D	D	D
PA8.1050	A	A	A	A	A	C	C	A	A	C	A	A	A	C	A	-	A	A	A	A	A	C
PA8.130	D	D	B	D	D	B	B	B	D	D	B	D	B	B	B	B	D	D	B	D	B	D
PA8.510	C	C	A	C	C	C	C	C	C	A	A	C	C	A	A	C	C	A	C	C	A	A
PA8.990	A	C	A	A	C	C	C	A	A	C	A	A	A	C	C	C	A	A	C	A	A	C
S2A7.120	C	C	C	A	A	A	C	C	C	C	A	A	A	C	C	C	C	A	C	C	A	A
S2A7.1500	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
S2A7.630	A	A	A	A	A	A	H	H	H	B	A	A	A	H	H	H	H	H	H	A	A	A
S2A7.690	D	D	B	D	D	B	D	D	D	D	B	D	D	D	B	D	D	D	B	D	D	D
S2A7.880	B	-	D	D	B	D	D	D	D	D	B	D	D	D	B	D	D	D	D	B	D	D
S2A7.980	A	C	C	C	A	C	C	C	C	C	C	C	C	C	A	C	-	C	C	C	A	C
S3A11.1590	D	B	B	D	D	D	D	D	D	D	B	D	D	D	D	D	D	B	B	D	D	D
S3A11.450	A	A	A	C	A	A	C	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
S3A11.490	D	D	B	D	D	D	D	D	D	D	D	D	D	D	B	D	D	D	D	D	B	D
S3A11.760	A	C	A	C	A	A	A	A	C	A	A	A	A	A	A	A	C	A	A	A	A	A
S3A11.850	C	C	C	C	A	C	C	C	C	C	C	A	A	C	C	C	A	A	C	C	C	C
S3A4.1550	D	B	B	D	D	D	D	D	D	D	B	B	D	D	B	D	D	B	B	D	D	D
S3A4.530	B	H	B	B	B	B	B	B	B	H	B	B	B	B	-	B	B	H	-	B	-	B
S3A4.660	D	D	B	D	D	B	D	D	B	B	D	D	B	B	D	D	B	B	D	B	B	D
S3A4.890	C	C	C	C	A	C	C	C	C	C	C	A	A	C	C	C	A	A	C	C	C	C

Score-sheet for TRAP markers (Cross: Tift $23D_2B_1$-P1-P5 × WSIL-P8; $F_{2:4}$ Genotypes = 97) contd.

S.No.	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97
$F_{2:4}$No.	122	123	124	125	127	130	131	132	133	135	136	137	138	139	140	145	147	148	151	153	159
PA1.805	D	B	D	D	D	D	B	D	B	B	D	D	B	D	D	B	D	B	D	B	D
PA1.910	C	A	C	C	C	C	C	C	A	A	C	A	C	C	A	A	A	A	C	C	C
PA1.990	C	A	C	A	C	C	A	C	A	C	C	C	C	C	A	A	A	C	C	C	C
PA15.1050	C	C	C	C	C	C	A	C	A	A	C	C	C	A	A	C	A	A	C	C	A
PA15.165	B	B	B	B	B	B	A	B	A	H	B	B	B	B	A	A	B	H	B	A	A
PA15.200	D	D	D	B	D	D	B	D	B	B	D	D	D	D	B	D	D	B	D	D	D
PA15.290	A	A	A	B	B	A	H	B	-	A	H	A	B	A	A	H	B	A	A	B	B
PA15.550	B	B	B	B	-	D	D	B	B	B	B	D	D	D	B	B	B	B	D	B	B
PA8.1050	C	A	A	A	C	A	A	C	C	C	C	A	C	C	A	A	A	A	C	C	A
PA8.130	B	B	B	B	B	D	B	D	B	D	D	D	B	B	D	B	B	B	D	D	D
PA8.510	C	C	C	C	C	C	C	A	A	A	C	A	A	A	C	A	A	A	C	A	A
PA8.990	C	A	A	A	C	A	A	C	C	A	A	A	C	C	A	A	A	A	C	A	A
S2A7.120	A	A	A	C	C	C	C	C	A	-	A	A	C	C	A	A	C	C	C	A	A
S2A7.1500	D	D	D	B	D	D	D	D	D	-	D	D	D	D	D	D	B	D	B	B	B
S2A7.630	B	B	H	H	H	H	B	H	H	-	H	B	A	A	H	H	-	H	H	B	A
S2A7.690	D	D	D	D	D	D	D	D	D	-	D	D	D	B	D	B	B	D	D	D	D
S2A7.880	D	D	D	B	D	D	D	D	D	-	D	D	D	D	B	B	B	D	D	B	B
S2A7.980	A	A	A	C	A	C	C	C	C	-	A	A	A	A	C	C	A	A	A	C	A
S3A11.1590	D	D	D	D	D	D	D	D	B	B	D	D	D	D	D	D	B	B	-	B	D
S3A11.450	C	C	C	C	A	A	C	C	A	A	C	C	A	A	A	C	A	A	-	C	C
S3A11.490	D	D	D	D	D	D	D	D	B	B	D	D	D	D	B	D	B	B	-	D	D
S3A11.760	C	C	C	C	A	C	C	C	A	A	C	C	A	C	A	C	A	A	-	A	A
S3A11.850	C	C	C	C	C	C	C	C	A	A	C	C	C	C	C	C	A	A	-	C	C
S3A4.1550	D	D	D	D	D	D	D	B	B	B	B	B	D	D	B	D	B	D	B	B	D
S3A4.530	B	B	B	H	B	B	H	-	H	-	B	B	-	B	A	-	B	-	H	H	B
S3A4.660	D	B	B	D	B	B	D	B	B	B	D	B	B	B	B	D	B	B	D	D	D
S3A4.890	C	C	C	C	C	C	C	C	A	C	A	C	C	C	C	C	A	C	A	A	A

Score-sheet for TRAP markers (Cross: Tift $23D_2B_1$-P1-P5 × WSIL-P8; $F_{2:4}$ Genotypes = 97) contd.

S.No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
$F_{2:4}$No.	7	11	14	15	16	19	20	21	24	25	26	27	28	29	30	31	32	33	35	36	37	38	39	41	43	45	47
S3A4.950	C	A	C	C	C	A	C	C	C	A	C	A	A	C	C	C	C	C	C	C	C	C	C	C	A	A	C
S5A8.260	C	A	C	C	C	A	C	C	C	C	A	C	A	A	C	A	C	A	A	C	A	C	A	A	C	C	C
S5A8.340	C	C	C	C	A	C	C	A	C	C	C	C	A	C	C	A	C	C	C	A	A	C	C	A	A	C	C
S5A8.490	C	C	A	A	C	C	C	A	C	A	C	A	A	A	A	A	A	A	A	C	A	C	A	A	A	A	A
S5A8.690	D	D	B	D	B	B	D	B	B	D	B	B	B	B	D	B	B	D	D	B	B	D	D	D	B	D	D
S5A9.110	B	D	D	D	D	B	D	D	D	D	D	B	B	B	B	D	B	D	D	D	D	B	D	B	B	B	B
S5A9.210	D	D	D	D	D	D	B	D	D	D	D	D	D	D	D	D	D	B	D	D	D	D	D	D	B	B	B
S5A9.410	C	C	A	C	C	C	A	A	C	A	C	C	C	C	C	C	C	C	A	C	C	C	A	C	C	C	A
S5A9.510	A	C	A	C	A	A	C	C	C	C	C	A	A	C	A	C	C	C	A	A	C	A	C	C	C	A	C
S5A9.700	D	B	D	D	B	D	B	D	B	B	D	D	D	B	D	D	B	B	D	D	B	D	B	D	B	D	D

S.No.	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54
$F_{2:4}$No.	48	49	51	53	55	56	62	63	64	69	70	72	73	74	75	76	77	78	79	80	81	82	84	85	86	87	88
S3A4.950	C	C	C	C	C	A	A	A	A	A	A	C	C	A	C	A	A	A	C	C	C	C	C	A	C	A	C
S5A8.260	C	A	C	A	A	A	A	C	A	A	C	A	A	C	C	C	C	A	A	A	A	A	C	A	C	C	A
S5A8.340	C	A	C	C	A	C	C	C	C	C	C	C	A	C	C	C	C	C	C	C	A	A	C	A	C	A	C
S5A8.490	A	C	A	C	A	C	C	C	C	A	A	C	A	C	A	C	A	C	A	A	C	C	A	C	C	C	A
S5A8.690	D	B	B	B	B	D	D	B	D	B	D	B	B	B	D	B	D	B	B	D	-	D	D	D	B	D	D
S5A9.110	B	D	D	D	D	D	D	D	D	D	D	B	D	B	B	B	D	D	D	D	B	B	D	B	D	D	B
S5A9.210	D	D	D	D	B	B	D	D	D	D	B	D	D	D	D	D	D	D	D	D	D	B	D	B	D	D	D
S5A9.410	A	C	C	A	A	A	C	C	A	A	A	C	C	C	C	C	C	C	C	C	C	A	C	A	C	C	A
S5A9.510	C	C	A	C	-	C	A	A	C	A	C	C	C	A	C	C	C	C	C	A	C	C	C	A	A	A	C
S5A9.700	B	D	D	D	D	B	D	D	B	D	D	-	D	D	B	D	D	D	B	D	D	D	B	D	D	D	B

Score-sheet for TRAP markers (Cross: Tift $23D_2B_1$-P1-P5 × WSIL-P8; $F_{2:4}$ Genotypes = 97) contd.

S.No.	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76
F_{24}No.	89	92	95	96	97	99	100	101	102	103	106	107	108	110	111	114	115	116	117	118	119	120
S3A4.950	C	C	A	C	A	C	C	C	C	C	A	A	A	C	C	C	C	C	C	A	A	C
S5A8.260	A	A	C	C	C	C	A	C	C	A	A	C	C	C	A	C	C	A	C	A	C	C
S5A8.340	A	A	A	A	C	A	A	A	A	A	C	C	A	C	A	C	C	A	C	C	C	C
S5A8.490	C	A	C	C	A	C	C	C	C	C	A	A	C	A	C	A	A	A	A	C	C	A
S5A8.690	D	B	B	D	D	D	B	D	B	B	D	D	B	D	B	D	B	B	D	D	D	B
S5A9.110	D	D	D	B	B	B	D	D	B	D	B	D	B	D	D	D	D	D	B	D	D	D
S5A9.210	D	D	B	B	D	D	D	B	B	D	D	D	D	B	D	D	B	D	D	D	D	D
S5A9.410	C	C	C	A	A	A	C	A	C	A	A	A	C	C	A	A	A	C	A	C	A	A
S5A9.510	A	C	C	A	A	C	A	C	A	C	C	A	A	C	C	C	C	A	C	C	A	A
S5A9.700	D	D	D	D	D	D	D	B	D	D	D	D	D	B	B	D	D	D	B	D	D	D

S.No.	77	78	73	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97
F_{24}No.	122	123	124	125	127	130	131	132	133	135	136	137	138	139	140	145	147	148	151	153	159
S3A4.950	A	C	C	C	C	C	C	A	A	C	C	C	A	C	C	C	A	C	A	C	A
S5A8.260	A	A	C	A	A	A	A	A	C	C	C	A	A	C	A	A	A	A	C	A	A
S5A8.340	C	C	A	A	A	C	A	C	A	C	C	A	C	C	C	A	C	A	C	C	A
S5A8.490	A	A	A	A	A	A	C	A	C	A	C	C	A	C	C	C	C	C	-	C	C
S5A8.690	D	D	D	B	B	B	D	D	B	B	D	B	B	D	B	D	D	B	B	D	B
S5A9.110	D	D	B	B	B	D	B	D	B	D	B	D	B	D	B	D	D	B	B	B	D
S5A9.210	D	B	D	D	B	D	D	D	B	B	D	D	D	D	D	D	B	D	B	B	B
S5A9.410	A	A	A	A	C	C	A	A	A	A	A	C	C	C	C	C	A	C	A	A	A
S5A9.510	C	C	C	C	C	A	C	C	C	A	A	C	C	C	A	C	A	C	A	A	A
S5A9.700	B	B	-	B	B	D	B	B	B	B	D	D	B	B	D	D	B	B	D	D	D

ERRATA

The following are the corrections incorporated or the justifications given for the suggestions made by external examiners:

1. The names of authors are re-arranged on the pages 14, 15, 17, 21, 25, 38, and 39 according to chronological order.
2. Table 4.9 could not fit into a single page if the parts A, B, C are merged into a single table. The parts A, B, C represent the values of sensitive, moderately tolerant and highly tolerant lines on a single page in portrait format for a comparative purpose of these lines at a glance. Therefore, the Table 4.9 is retained as it is.
3. The significance of increase in proline content in all the lines (including salt-tolerant lines) was tested statistically. The standard errors and the values of significance are given in parentheses and footnotes.
4. The format of Table 4.10 could not be modified or retyped into a single page in any way for comparison of ions due to space limitations. Further, the ion uptake in each line for Na^+, K^+, Ca^{2+} and Cl^- relative to each other becomes clear in each sub-part of Table 4.10, representing ion content in each line. Hence, this table is not modified.