



**UNIVERSITI PUTRA MALAYSIA**

**ISOLATION AND SEQUENCE ANALYSES OF  
SALINITY TOLERANCE GENES FROM BRUGUIERA  
CYLINDRICA (L.) BLUME**

**WONG YEEN YEE**

**FBSB 2005 17**

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**By**

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

**July 2005**



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

**ISOLATION AND SEQUENCE ANALYSES OF SALINITY TOLERANCE  
GENES FROM *BRUGUIERA CYLINDRICA* (L.) BLUME**

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**July 2005**

**Chairman : Ho Chai Ling, PhD**

**Faculty : Biotechnology and Biomolecular Sciences**

Salinity is a major abiotic stress limiting the productivity of crop plants globally. The discovery of novel genes in stress adaptation will provide effective genetic engineering strategies leading to greater stress tolerance. The objectives of this research are to identify and isolate salinity tolerance genes from the mangrove plant, *Bruguiera cylindrica* (L.) Blume through suppression subtractive hybridization (SSH) and bacterial functional assay.

*B. cylindrica* propagules were grown in fresh water and 20 ppt salinity water. Root morphology differences between *B. cylindrica* grown in fresh water and 20 ppt salinity water were largely due to the need of roots to obtain more water and nutrients during salinity stress. *B. cylindrica* plants grew better in the presence of salt as higher mean values were obtained for all morphological measurements compared to *B. cylindrica* plants grown in fresh water.

Four RNA extraction methods were attempted to obtain high yield and high purity RNA. The cesium chloride method was chosen for RNA extraction as it gave the highest amount of pure RNA. Subtracted cDNAs were prepared from the roots of the *B. cylindrica* seedlings that were grown in fresh water and salt water, respectively. A total of 84 subtracted cDNAs were cloned into pCR-BLUNT II TOPO and sequenced. A total of 51 subtracted cDNAs with good sequencing quality were assembled into 7 contigs and 10 singletons. These non-redundant sequences were grouped into unknown protein (41.18%), novel (29.41%), protein destination and storage (11.76%), energy (5.88%), intracellular traffic (5.88%) and protein synthesis (5.88%). Some motifs of novel and unknown sequences may involve in the salinity tolerance of *B. cylindrica* such as Kv1.3 voltage-gated K<sup>+</sup> ion(s) channel signature, calcium-activated BK potassium channel alpha subunit and Kir2.1 inward rectifier K<sup>+</sup> ion(s) channel signature.

Meanwhile, a cDNA library was also constructed from the roots of *B. cylindrica* that were grown in fresh water. Bacterial functional assay was performed to identify cDNAs that confer salt tolerance. A total of 85 cDNA clones that were able to grow on 2× YT containing 400 mM NaCl were sequenced and 73 cDNAs with good sequence quality were assembled into 9 contigs and 53 singletons. The non-redundant sequences were also categorised into unknown protein (58.06%), metabolism (9.68%), transporters (9.68%), transcription (6.45%), energy (4.84%), cell growth/division (4.84%), novel (3.23%), miscellaneous (1.61%) and disease/defense (1.61%). A motif search on novel and unknown cDNA

sequences had revealed some possible motifs that may be involved in salinity tolerance of *B. cylindrica* e.g. *C. elegans* Srg family integral membrane protein signature and 2Fe-2S ferredoxins, iron-sulfur binding region signature.

Sequence analysis of subtracted cDNAs and putative salt tolerant cDNAs isolated by bacterial functional assay showed some putative proteins that may be involved in the salinity tolerance of *B. cylindrica* such as putative potassium transporter HAK1p (M33), putative zinc finger protein (M3), ubiquitin (BC27) and L-ascorbate peroxidase (A46).



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

**PEMENCILAN DAN ANALISIS TURUTAN GEN-GEN TOLERANSI  
KEMASINAN DARI *BRUGUIERA CYLINDRICA* (L.) BLUME**

Oleh

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Kemasinan merupakan suatu tekanan abiotik utama yang menghadkan produktiviti tanaman di seluruh dunia. Penemuan gen-gen novel yang terlibat dalam adaptasi tekanan persekitaran akan menyediakan asas strategi pengubahsuaian genetik ke arah penyesuaian tanaman terhadap tekanan persekitaran yang lebih tinggi. Matlamat penyelidikan ini adalah untuk mengenalpasti dan memencilkan gen-gen dalam toleransi kemasinan dari pokok bakau, *Bruguiera cylindrica* (L.) Blume melalui strategi “suppression subtractive hybridization (SSH)” dan “bacterial functional assay”.

Biji benih vivipariti *B. cylindrica* disiram air biasa dan air bergaram sebanyak 20 ppt. Perbezaan morfologi akar yang terhasil di antara *B. cylindrica* yang disiram dengan air biasa dan air bergaram kemungkinan disebabkan oleh keperluan akar untuk memperolehi air dan nutrien yang lebih banyak semasa berada di dalam keadaan tekanan kemasinan. *B. cylindrica* membesar dengan lebih baik dengan kehadiran garam kerana nilai purata

ukuran morfologi yang lebih tinggi diperolehi dibandingkan dengan *B. cylindrica* yang disiram dengan air biasa.

Daripada empat kaedah pengekstrakan RNA yang telah dicuba, kaedah cesium klorida telah dipilih kerana kaedah ini memberi hasil RNA yang paling tulen dan tinggi. cDNA yang diekspres di dalam akar *B. cylindrica* yang membesar dengan kehadiran garam sahaja telah diperolehi melalui strategi SSH. Strategi SSH menyingkirkan cDNA di dalam akar *B. cylindrica* yang disiram dengan air biasa. Sejumlah 81 klon dari SSH telah diklon ke dalam pCR-BLUNT II TOPO dan diujuk. Sebanyak 51 jujukan cDNA yang berkualiti dihimpunkan ke dalam 7 “contig” and 10 “singleton”. Jujukan-jujukan yang tidak redandensi ini dikumpulkan ke dalam kumpulan protin tidak diketahui (41.18%), unik (29.41); destinasi and penyimpanan protin (11.76%), tenaga (5.88%), trafik intrasel (5.88%) dan protin sintesis (5.88%). Motif yang dimiliki oleh jujukan unik dan tidak diketahui mungkin terlibat di dalam toleransi kemasinan pokok bakau *B. cylindrica* seperti Kv1.3 voltage-gated K<sup>+</sup> ion(s) channel signature, calcium-activated BK potassium channel alpha subunit and Kir2.1 inward rectifier K<sup>+</sup> ion(s) channel signature.

Selain itu, satu perpustakaan cDNA telah disediakan daripada akar *B. cylindrica* yang disiram dengan air biasa. “Bacterial functional assay” telah digunakan untuk mengenalpasti cDNA yang mempunyai sifat toleransi kemasinan. Sejumlah 85 klon yang tumbuh di atas plat 2× YT telah dipencil dan diujuk. Sebanyak 73 jujukan yang berkualiti dihimpunkan ke dalam 9 “contig” and 53 “singleton”. Jujukan-jujukan yang tidak redandensi turut

dikategorikan ke dalam kumpulan protin tidak diketahui (58.06%), metabolime (9.68%), pengangkutan (9.68%), transkripsi (6.45%), tenaga (4.84%), pertumbuhan and pembahagian sel (4.84%), unik (3.23%), lain-lain (1.61%) serta penyakit dan pertahanan (1.61%). Carian motif ke atas jujukan unik dan tidak diketahui telah memberi gambaran terhadap motif yang mungkin terlibat di dalam toleransi kemasinan *B. cylindrica* seperti *C. elegans* Srg family integral membrane protein signature and 2Fe-2S ferredoxins, iron-sulfur binding region signature.

Analisa penjujukan cDNA dari SSH dan "bacterial functional assay" menunjukkan kehadiran protin-protin yang mungkin terlibat di dalam toleransi kemasinan pokok bakau, *B. cylindrica* seperti "putative potassium transporter HAK1p" (M33), "putative zinc finger protein" (M3), "ubiquitin" (BC27) and "L-ascorbate peroxidase" (A46).



## ACKNOWLEDGEMENTS

I would like to express my gratitude and appreciation to Dr. Ho Chai Ling for giving me the opportunity to pursue my studies in the field of molecular biology. Throughout my study period, she provided encouragement, sound advice, good teaching and also for her patience in guiding me. Not to forget Assoc. Prof. Dr Jenni, Assoc. Prof. Dr. Raha and Dr Michael Wong for their guidance and advices throughout my study.

Special thanks to my lab mates: Dr. Sudha, Tony, Seddon, Dang, Swee Sen and many others for their kind assistance, advices and support. I am grateful to Cheong Xin for his help in journals searching; Mr. Lee Weng Wah and Mr. Lee Yang Ping for answering all my queries in sequence analysis.

I would also like to acknowledge the Ministry of Science, Technology and Innovation, Malaysia, for providing financial support of PASCA postgraduate fellowship.

Lastly, and most importantly, I wish to thank my family for their understanding, love and support. To them I dedicate this thesis.



I certify that an Examination Committee met on 25<sup>th</sup> July 2005 to conduct the final examination of Wong Yeen Yee on her Master of Science thesis entitled "Isolation and Sequence Analyses of Salinity Tolerance Genes from *Brugeiera cylindrical* (L.) Blume" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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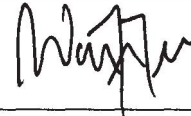
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## DECLARATION

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



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**WONG YEEN YEE**

Date: 19/10/05

## TABLE OF CONTENTS

	Page
<b>ABSTRACT</b>	ii
<b>ABSTRAK</b>	v
<b>ACKNOWLEDGEMENTS</b>	viii
<b>APPROVAL</b>	ix
<b>DECLARATION</b>	xi
<b>LIST OF TABLES</b>	xv
<b>LIST OF FIGURES</b>	xvi
<b>LIST OF ABBREVIATIONS</b>	xviii
<b>CHAPTER</b>	
<b>1 INTRODUCTION</b>	<b>1</b>
<b>2 LITERATURE REVIEW</b>	<b>3</b>
2.1 Stress in plants	3
2.1.1 Salt and drought stress	3
2.2 Stress tolerance in plants	7
2.2.1 Salt tolerance in plants	8
2.3 Mechanism of salt tolerance in plants	9
2.3.1 Detoxification	11
2.3.2 Homeostasis	12
2.3.3 Growth regulation	22
2.4 Mangroves	23
2.4.1 Importance of mangroves	25
2.4.2 <i>Bruguiera cylindrica</i> (L.) Blume	29
2.4.3 Salt tolerance in <i>Bruguiera</i> sp.	33
2.5 Differentially expressed genes	36
2.5.1 Other methods	39
2.6 Functional assay	42
<b>3 MATERIALS AND METHODS</b>	<b>46</b>
3.1 Sample collection and preparation	46
3.2 Optimization of RNA extraction	47
3.2.1 Method 1: Cesium chloride method (Glišin <i>et al.</i> , 1974)	47
3.2.2 Method 2: CTAB (hexacetyltrimethyl ammonium bromide) method (Apt <i>et al.</i> 1995)	49
3.2.3 Method 3: CTAB (hexacetyltrimethyl ammonium bromide) method (Apt <i>et al.</i> 1995) with potassium acetate	50
3.2.4 Method 4: CTAB (hexacetyltrimethyl ammonium bromide) method (Sugihara <i>et al.</i> 2000)	50
3.3 Isolation of mRNA from total RNA	51
3.4 Suppression subtractive hybridization (SSH)	52
3.4.1 First-strand cDNA synthesis	52

3.4.2	Second-strand cDNA synthesis	52
3.4.3	<i>RsaI</i> digestion	53
3.4.4	Adaptor ligation	54
3.4.5	First hybridization	55
3.4.6	Second hybridization	55
3.4.7	Primary PCR amplification	55
3.4.8	Secondary PCR amplification	56
3.5	Analysis of subtracted cDNAs	56
3.5.1	Purification of subtracted cDNAs	56
3.5.2	Preparation of pCR®-Blunt II-TOPO plasmid	57
3.5.3	Ligation of subtracted cDNAs to pCR®-Blunt II-TOPO	58
3.5.4	Transformation of ligated plasmid into TOP10 competent cell	58
3.5.5	Preparation of plasmid DNA	58
3.5.6	Restriction enzymes analysis of transformants	60
3.5.7	PCR analysis of transformants	60
3.5.8	Sequencing	60
3.5.9	Sequence analysis	61
3.6	Yeast functional assay	62
3.6.1	Preparation of pYC2/NT A, B and C	62
3.6.2	Ligation of subtracted cDNAs to pYC2/NT A	65
3.6.3	Transformation of ligated plasmid into TOP10 cells	65
3.6.4	Preparation of plasmid DNA	65
3.6.5	Restriction enzymes analysis of transformants	65
3.6.6	PCR analysis of transformants	66
3.6.7	Sequencing of positive clones	66
3.6.8	Yeast transformation of ligated plasmid into InVSc1 cells	66
3.6.9	PCR confirmation of yeast transformants	67
3.6.10	Salt tolerance testing on yeast transformants	68
3.7	Construction of cDNA library from <i>B. cylindrica</i> roots	68
3.7.1	First-strand cDNA synthesis	69
3.7.2	Second-strand cDNA synthesis	69
3.7.3	Blunting the cDNA termini	69
3.7.4	Ligation of <i>EcoRI</i> adapters	70
3.7.5	Phosphorylating the <i>EcoRI</i> ends	70
3.7.6	<i>XhoI</i> digestion	71
3.7.7	Size fractionation	71
3.7.8	Quantification of cDNA with ethidium bromide	72
3.7.9	Ligation of cDNA to Uni-ZAP XR vector	72
3.7.10	Preparing the host bacteria	72
3.7.11	Packaging the ligated product	73
3.7.12	Primary plating and titering	73
3.7.13	<i>In vivo</i> excision	74
3.7.14	Amplification of cDNA library	75
3.8	Bacterial functional assay	76
3.8.1	Mass Excision	76
3.8.2	Plating	77



<b>4</b>	<b>RESULTS</b>	78
4.1	Sample collection and preparation	78
4.2	RNA extraction	81
4.3	Isolation of mRNA from total RNA	84
4.4	Suppression subtractive hybridization (SSH)	84
4.5	Yeast functional assay	92
4.6	Bacteria functional assay	96
<b>5</b>	<b>DISCUSSION</b>	106
5.1	Sample collection and preparation	106
5.2	RNA extraction	110
5.3	Suppression subtractive hybridization (SSH)	111
5.4	Yeast functional assay	115
5.5	Bacteria functional assay	115
5.6	The possible salinity tolerance genes in <i>B. cylindrica</i>	118
<b>6</b>	<b>CONCLUSION</b>	123
6.1	Recommended study	124
	<b>REFERENCES</b>	126
	<b>APPENDICES</b>	148
	Appendix A	148
	Appendix B	150
	Appendix C	151
	Appendix D	152
	Appendix E	153
	Appendix F	158
	Appendix G	162
	Appendix H	163
	Appendix I	164
	Appendix J	172
	Appendix K	186
	Appendix L	187
	Appendix M	188
	<b>BIODATA OF THE AUTHOR</b>	189



## LIST OF TABLES

Table		Page
1	Sources of environmental stress for plants (reproduced from Hale and Orcutt, 1987)	4
2	Products of mangrove ecosystem (reproduced from Sanger <i>et al.</i> , 1983)	28
3	Measurements of <i>B. cylindrica</i> plants grown in green house in fresh water and 20 ppt salinity water, respectively	80
4	A comparison of results from four RNA extraction methods	82
5	The assembled contigs of <i>B. cylindrica</i> subtracted cDNAs	89
6	Putative functions of subtracted cDNAs	91
7	Subtracted cDNAs that had been successfully ligated to pYC2/NT	95
8	The assembled contigs from the putative salt tolerant cDNAs of <i>B. cylindrica</i> roots that were isolated by bacterial functional assay	102
9	Putative functions of the salt tolerant cDNAs of <i>B. cylindrica</i> roots that were isolated by bacterial functional assay	104





## LIST OF FIGURES

Figure		Page
1	Functional demarcation of salt and drought stress signaling pathways (reproduced from Zhu, 2002).	10
2	Na <sup>+</sup> ion(s) uptake and extrusion at the plant plasma membrane (reproduced from Blumwald <i>et al.</i> , 2000).	17
3	Regulation of ion (e.g., Na <sup>+</sup> ion(s) and K <sup>+</sup> ion(s)) homeostasis by the SOS pathway (reproduced from Zhu, 2003).	17
4	Na <sup>+</sup> ion(s) compartmentation at the tonoplast (reproduced from Blumwald <i>et al.</i> , 2000).	21
5	Flowers of <i>B. cylindrica</i> (reproduced from Tan, 2001).	31
6	<i>B. cylindrica</i> plants at Morib, Selangor Darul Ehsan (source: Wong Yeen Yee).	31
7	<i>B. cylindrica</i> roots with kneed pneumatophores (loops protruding out of the sand) (reproduced from Ng and Sivasothi, 2001).	32
8	Young propagule of <i>B. cylindrica</i> at Morib, Selangor Darul Ehsan (source: Wong Yeen Yee).	32
9	Schematic diagram of PCR-Select cDNA subtraction (reproduced from Clontech PCR-Select™ cDNA Subtraction kit user manual, 2002).	38
10	Morphology of 4 month-old <i>B. cylindrica</i> seedlings grew in fresh water and 20 ppt salinity water.	79
11	RNA extracted from <i>B. cylindrica</i> roots by using method 1, method 2, method 3 and method 4.	83
12	Gel electrophoresis of subtracted cDNA library of roots of <i>B. cylindrica</i> grown in fresh water and 20 ppt salinity water.	86
13	Assembly of subtracted cDNAs into contigs using CAP3 sequence assembly program.	88
14	Graphic representation of sequences assembled in contig1.	90
15	The functional classification of 17 non-redundant subtracted cDNAs from the roots of <i>B. cylindrica</i> .	90

16	Growth of yeast strain INVSc1 with different OD <sub>600</sub> readings on YP induction plates with different concentrations of NaCl.	94
17	Gel electrophoresis of the PCR products of cDNA clones from the roots of <i>B. cylindrica</i> that grew in fresh water.	97
18	Mass-excised cDNA library of <i>B. cylindrica</i> roots plated on 2×YT plate containing 171 mM of NaCl and 400 mM of NaCl.	97
19	Putative salt tolerant colonies that were restreaked on 2× YT (400 mM) for reconfirmation.	98
20	Assembly of cDNAs sequence obtained from bacterial functional assay into contigs using CAP3 sequence assembly program.	101
21	Graphic representation of putative salt tolerant cDNA sequences in contig 6 obtained from bacterial functional assay.	103
22	The functional classification of 62 non-redundant putative salt tolerant cDNA clones of <i>B. cylindrica</i> roots.	103
23	Root morphology of <i>Arabidopsis</i> in high and low concentration of phosphorus, respectively; compared with root morphology of <i>B. cylindrica</i> grown in fresh water and 20 ppt salinity water, respectively	109



## LIST OF ABBREVIATIONS

$\beta$	beta
$\lambda$	lambda
$\times g$	gravitational acceleration
$\mu g$	microgram
$\mu L$	microliter
$^{\circ}C$	degree centigrade
%	percentage
AMV	avian myeloblastosis virus
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BSA	bovine serum albumin
Ca	calcium
cDNA	complementary DNA
CIP	calf intestinal phosphatase
Cl	chloride
cm	centimeter
CsCl	cesium chloride
CTAB	hexacetyltrimethyl ammonium bromide
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
DEPC	diethyl pyrocarbonate
dGTP	2'-deoxy-guanosine-5'-triphosphate
DNA	deoxyribonucleic acid



DNase	deoxyribonuclease
dNTPs	deoxynucleotides
ds	double-stranded
DTT	dithiothreitol
dTTP	thymidine-5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
g	gram
HCl	hydrochloric acid
K	potassium
kb	kilo base pairs
L	liter
LB	Luria-bertani
LiCl	lithium chloride
M	molar
Mg	magnesium
MgSO <sub>4</sub>	magnesium sulfate
mL	milliliter
mM	millimolar
mRNA	messenger RNA
Na	sodium
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information



ng	nanogram
NH <sub>4</sub> Oac	ammonium acetate
OD	optical density
PCR	polymerase chain reactions
<i>pfu</i>	plaque forming units
ppt	part per thousand
RNA	ribonucleic acid
RNase A	ribonuclease A
ROS	reactive oxygen species
rpm	revolution per minute
SC-U	SC minimal medium without uracil
SDS	sodium dodecyl sulphate
SOS	salt overly sensitive
SSH	suppression subtractive hybridization
U	units
v/v	volume per volume
w/v	weight per volume
YPD	yeast extract peptone dextrose medium
YT	Yeast extract tryptone medium



# CHAPTER 1

## INTRODUCTION

Salinity is one of the major abiotic stress limiting plant productivity and growth globally. Salinity imposes osmotic stress and ionic stress to the plants. Irrigation practices in agricultural lands have steadily increased the concentration of salt in the soil (Khan and Duke, 2001). Unfavorable physiochemical environments can cause average losses more than 65% of optimal yields (Boyer, 1982). More efficient and productive agriculture will be possible on salt affected soils if crop plants with improved salinity tolerance can be selected and bred through traditional breeding or genetic engineering.

As halophytes can live under high salinity condition, it is advantageous to identify genes that are involved in the salinity tolerance of these plants to adapt to harsh environment. Mangroves are unique communities along the tropical and sub-tropical coastal regions that are formed by almost fifty unrelated plant species (Banzai *et al.*, 2002a). Mangroves are divided into two groups based on their morphological features of salt management i.e. 'secreters' and 'non-secreters' (Tomlinson, 1986). The 'secreters' possess salt glands or salt hairs to eliminate excess salt from plants while the 'non-secreters' have no such morphological devices (Tomlinson, 1986; Banzai *et al.*, 2002a).

In this study, *Bruguiera cylindrica* (L.) Blume, known locally in Malaysia as "bakau putih", was chosen as a source to study novel salinity tolerance



genes in halophytes. *B. cylindrica* is categorized as one of the 'non-secreters' and it tolerates high salinity through the ultrafiltration system whereby the plant is able to exclude a large proportion of salt from the water it uptakes and selectively absorbs only certain ions (Tomlinson, 1986).

The objectives of this study were to identify genes that are involved in salinity tolerance of *B. cylindrica* using suppression subtractive hybridization (SSH) and bacterial functional assay. By using the SSH method, genes that were expressed only in the root of *B. cylindrica* grown in the presence of NaCl can be identified. Meanwhile, *B. cylindrica* cDNAs that confer salt tolerance to bacteria can be isolated by performing bacterial functional assay.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Stress in plants

Stress results changes in plant physiology caused by one or more environmental and biological factors (Hale and Orcutt, 1987). Table 1 shows the sources of environmental stress for plants (Hale and Orcutt, 1987). Stresses are divided into two groups i.e. abiotic and biotic stress. Abiotic stress depends on geographical and climatic differentiation such as annual rainfall differences, chilling, heat, drought, salinity, flooding and freezing (Holmberg and Bülow, 1998; Abeysinghe *et al.*, 2000). Whereas, examples of biotic stresses are diseases and pests.

##### 2.1.1 Salt and drought stress

Salinity and drought are the two major environmental factors that reduce plant productivity currently (Serrano *et al.*, 1999). Boyer (1982) mentioned that disease and insect damages cause losses of less than 10% while unfavorable physiochemical environments cause losses of more than 65%. From historical records, civilizations had never progressed in one locality for more than 1000-2000 years because of the destruction of the resource base of the area (Ashraf, 1994). As a result of poor water management, civilizations have been destroyed by the accumulation of salt on the soils. If there is limited rainfall, salt is not leached out of the soil. Crop plants take in salt through their roots and yields are reduced as the salt concentration increases. Drought and salinity are interconnected because crop production



Table 1. Sources of environmental stress for plants (reproduced from Hale and Orcutt, 1987).

Physical	Chemical	Biotic
Drought	Air pollution	Competition
Temperature	Allelochemicals	Allelopathy
Radiation	(organic)	Lack of symbioses
Flooding	Nutrients (inorganic)	Human activities
Mechanical	Pesticides	Diseases
Electrical	Toxins	Insects
Magnetic	Salts	
Wind	pH of soil solution	

