University of Southampton

Development of Drought Resistant Varieties of Rice (*Oryza sativa* L.) Through *In Vitro* Hybridization

Mohammad Khalequzzaman

A thesis submitted for the degree of Doctor of Philosophy

Faculty of Engineering and Applied Science
Department of Civil and Environmental Engineering
University of Southampton
United Kingdom

September 2002

Dedicated to

the departed soul of my father

Kabi Mofizuddin Ahmed

Whose dream was that

I should complete my studies in England

Acknowledgements

All my thanks go to the almighty Allah for giving me ability to complete the study.

I gratefully acknowledge the Government of the People's Republic of Bangladesh for their financial help of the study and my parent institute, Bangladesh Rice Research Institute for nominating me for the fellowship.

I would like to extend my thanks to my supervisor Dr. Nazmul Haq for his guidance, supervision and support in various ways.

My sincere thanks to Dr. D. Clarke for data analysis and his suggestion.

I gratefully acknowledge Dr. Hamid Miah, former Director General of the Bangladesh Rice Research Institute, Gazipur, Dr. Siddique of the Bangladesh Agricultural Research Council, Dhaka and Dr. M.A. Khaleque Mian of Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur for their help and co-operation.

My thanks to colleagues at the University of Southampton, especially the technical staff in the laboratory and glasshouse.

My individual thanks to Dr. N. Chowdhury, Dr. M. Bazle, Dr. M. A. Ali, Mr. Kyei-Baffour, Mr. Shah, Mr. Tariq and John for their various help.

My earnest thanks to all my friends and colleagues from home and abroad who helped directly or indirectly during the study.

My sincere gratitude to my mother *Fazilatunnesa*, whom I miss a lot, and also to all my brothers and sisters, sisters-in-law, brothers-in-law, and all other relatives for their blessing, affection, support and encouragement.

A final word of thanks goes to my long suffering wife Farzia Ahmed Shimmi and my beloved daughter Tazrian Zaman Tushti for their extreme patience and support.

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF ENGINEERING AND APPLIED SCIENCE
DEPARTMENT OF CIVIL AND ENVIRONMENTAL ENGINEERING

Doctor of Philosophy

Development of Drought Resistant Varieties of Rice (*Oryza sativa* L.) Through *In Vitro* Fertilization

by Mohammad Khalequzzaman

Drought is one of the major constraints to the productivity of rice in many parts of the world, particularly in South and Southeast Asia. The breeding for improved drought resistance types of rice has been slow. The aim of this study was to identify drought resistant accessions of rice and to investigate suitable methods for transfer of traits or genes in existing high yielding varieties through *in vitro* fertilization. Fifty-one rice accessions obtained from the Bangladesh Rice Research Institute (BRRI) were used to identify drought resistant accessions. Significant variations were observed in physio-morphological traits, such as drought score, root shoot length, root shoot dry weight, leaf stomata conductance, stomata number and water use efficiency. Most of the traits studied showed a significant correlation ($p<0.01$ and $p<0.05$) and some parameters appeared to be interrelated. Cluster analysis on physio-morphological characteristics has produced 4 to 6 discrete groups of the accessions. The analysis revealed that the most promising accessions for drought resistance were at least four and these were found to be in the same group.

To confirm the physio-morphological findings an analysis of 51 accessions using five isozyme systems was carried out. Maximum polymorphism was found to be with the esterase enzyme systems. Cluster analysis on 5-isozyme investigations also revealed 5 major groups. However, both cluster analyses of isozyme and physio-morphological data showed that 2 accessions could be identified as potential drought resistance types from these studies.

In order to transfer desirable traits to high yielding varieties of rice, attempts were made to develop methods for *in vitro* isolation of sperm and egg cells and their in vitro fertilization. Unfertilised ovules were macerated with different concentrations of cellulase (0.55 to 2.0%) and pectinase (0.55 to 1.50%) to determine the best concentration of enzymes for isolation of egg cells from embryo sacs. The ovules were incubated at 30°C in cell wall degrading maceration media, leading to their disintegration into loose cells. The best yield (35%) of egg cells was achieved when 0.04% (w/v) calcium chloride, 1% (w/v) cellulase and 0.75% pectinase were used. Fluorescein diacetate staining was used to determine the rate of viability of egg cells.

For isolation of sperm cells, different media compositions were used to maximise the yield of sperm cells. The best yield (17.8%) of viable sperm cells was obtained when pollen was cultured in a medium containing 0.008% (w/v) boric acid, 0.01% (w/v) potassium phosphate, 0.04% (w/v) calcium chloride and 20% (w/v) sucrose, 5 mM MOPS with a pH of 6. Sperm cells were separated from pollen remnants by infiltrating them after centrifugation on a percoll gradient. Fluorescein diacetate staining was used to determine the viability of sperm cells. A longer time of viability was obtained when vitamin-E (0.1 mM) and ascorbic acid (10 mM) were used in the medium. The results indicate that isolation of both sperm and egg cells is possible.

In vitro fusion or fertilization of rice sperm and egg cells was attempted. Different concentrations of calcium chloride (4 mM to 15mM) and pH (5 to 11) were used for *in vitro* fusion. The best fusion was obtained (55.56%) when a calcium chloride concentration was used at 7 mM along with other ingredients at a pH of 7.5. This method could be useful for direct gene transfer through regeneration of plants from fused gametes, not only in *Oryza* but also in other crop species.

ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
ACP	Acid Phosphatase
ADH	Alcohol Dehydrogenase
ANOVA	Analysis of Variance
BRRI	Bangladesh Rice Research Institute
CAT	Catalase
cm	centimetre
CV	Coefficient of Variation
DNA	De-oxyribonucleic acid
DMRT	Duncan Multiple Range Test
E.C.	Enzyme Commission
EST	Esterase
ET	Evapotranspiration
FAO	Food and Agricultural Organization
FDA	Fluorescein Diacetate
g	Gram
GOT	Glutamate Oxaloacetate Transaminase
GPI	Glucose-6-phosphate isomerase
HCA	Hierarchical Cluster Analysis
IRRI	International Rice Research Institute
kg	Kilogram
M	Molar
MDH	Malate Dehydrogenase
MES	2-[N-Morpholino]ethanesulfonic acid
mg	Milligram
mM	Milimolar
MOPS	3-[N-Morpholino]propanesulfonic acid
MS	Murashige and Skoog medium
MTT	(3-[4,5-Dimethyl thiazol-2-yl]-2,5-transaminase diphenyl tetrazolium bromide
NAA	α-Naphthalene acetic acid
NAD	β-Nicotinamide adenine dinucleotide
PCA	Principal Component Analysis
PGI	Phosphoglucoisomerase
PMS	Phenazine methosulphate
PRX	Peroxidase
PVP	40-Polyvinylpyrrolidone 40,000
r	Correlation coefficient
rf	Relative front
R^2	Coefficient of determination
RAPD	Random Amplified Polymorphic DNA
RCBD	Randomised Complete Block Design
RNA	Ribonucleic acid
SE	Standard Error of Mean
TEMED	N,N,N,'N,'-tetramethyl-ethylenediamine
UNO	United Nations Organization
WUE	Water use efficiency

TABLE OF CONTENTS

Index of Tables

Index of Figures

Index of Plates

Index of Appendices

CHAPTER ONE

1.1 General introduction

Demand for food and fibre is basically determined by the number of people to be fed and clothed. According to the United Nations Organization (UNO), the world's population is expected to rise to at least 9.3 billion by the year 2050 (UNO, 2001) and global demographic trends are not expected to stabilise before 2100. A special impact will be felt in developing countries, as projections indicate a rise in populations to 8.2 billion by 2050 from the present (2000) level of 4.9 billion. About 59% of the increase over the next 25 years will take place only in South Asia and sub-Saharan Africa (Table 1.1), where poverty and hunger are already widespread. As a result, more food and fibres are needed to satisfy demand, these being the most basic human needs.

Table 1.1 Population growth and increase in food requirements for different regions in the world, 2000-2025

Regions	Population (billions)		Projected per capita consumption of cereals (kg/yr.)		Food grain requirements (million tons)		Percentage increase 2000-2025
	2000	2025	2000	2025	2000	2025	
East Asia	1.48	1.70	284	332	420	564	34
South-central Asia	1.50	2.10	167	187	250	392	57
Southeast Asia	0.52	0.69	210	242	109	167	53
Western Asia and North Africa	0.36	0.55	405	469	146	258	77
Sub-Saharan Africa	0.65	1.20	138	156	90	187	108
Latin America	0.52	0.69	273	301	142	208	46
Developing Countries	4.90	6.82	258	280	1265	1910	51
Developed Countries	1.19	1.22	626	680	745	830	11

(Adapted from Hossain, 2001; UNO, 2001)

Cereal is a particularly important type of food, and yet per capita cereal consumption in the developing countries is low compared to the developed countries (Table 1.1) and this has special implications in sub-Saharan Africa and South Asia (Hossain, 2001). It is projected that within the next 25 years, the food requirement in sub-Saharan Africa will be doubled, and demand will increase by 50-75% (Table 1.1) in other parts of the developing countries. This means that an increase in food production of more than 50% is needed to meet the requirements of the projected population increase (James, 2000; UNO, 2001), which is one of the most formidable challenges for mankind. Moreover, food demand also increases from the changes in food habits of the people. This increased demand can only be achieved by diversifying crop production or by increasing crop productivity.

Rice (*Oryza sativa* L) is one of the world's most important food crops; it is cultivated in about 11% of the world's lands. Present annual rice production is 598.8 million tons, which is produced from about 153.7 million hectares of land (FAO, 2001). However, production of rice should increase by about 50% to 950 million tons by the year 2025 (out of a total cereal need of 2740 million tons Table 1.1) to keep up with population growth. Rice represents about 23% of global calorie consumption whereas wheat and maize represent only 17% and 9% respectively. Approximately 92 per cent (perhaps more) of the world's rice is produced and consumed in Asia, particularly in South and Southeast Asia, as a staple food, providing between 30-76% of the necessary calories per person (Khush, 2001). This shows that production and consumption of rice is mostly confined to Asia where the population density is high.

Rice, one of the most important carbohydrate sources for the world's population, is also used in the making of baby food, beer, soups, puddings and other food industries. In addition, rice by-products are used as fuel, feed, fertilizer, building materials and absorbents. Furthermore, rice bran is used as stock and poultry feed and in edible oil extraction (Turner and McCauley, 1983).

As far as increased food production is concerned, this can be achieved either by expanding cultivable lands or by increasing production per unit area. Evidence suggests that increased production per unit area is more promising than the expansion of land under cultivation (Paterson *et al.*, 1991; Toenniessen, 1991; Mannion, 1995). With growing urbanization and industrialisation, the amount of fertile agricultural farm-land used for the production of food and fibres has been decreasing, due to the demands for more and more housing, roads and factories. Moreover, the chances of increasing cultivable land are slim in densely populated areas of the world, such as Bangladesh and China. This being the case, high yielding varieties with excellent grain quality, adaptable to adverse agro-climatic conditions, such as drought, salinity, unsuitable soil structure, and nutrient deficiency with durable resistance to insects and pests are clearly required (Toenniessen, 1991). In other words, food production increase must be achieved from less land with less labour, water, fertilizer and pesticides, and the increase in production must be sustainable (Virmani, 1996). However, it is not an easy task to meet the demand for increased agricultural productivity with a decrease in the farm-land available. As a consequence, genetic improvement of crops represents one of the most important approaches in the struggle to attain a balance between food production and population growth (Khush, 1997; Tanksley and McCouch, 1997). The combined application of biotechnology and conventional breeding methods will relieve the task of increased food production. This research project has been undertaken to identify rice accessions with drought resistant trait(s), which are to be used in the rice improvement programmes.

1.2 The origins, classification and distribution of rice

Rice has been cultivated in the world for more than 7000 years, probably originating in between 10,000 and 15,000 years ago (Datta, 1981; Mannion, 1995). Its origins are always a matter of conjecture due to its diversified domestication and there are many different opinions in this regard. There is a possibility that domestication took place in several different centres at different times. It has also been reported by Chandraratna (1964) that several taxonomists ascribed different centres of origin of *O. sativa* on account of its wide range of variation. In general, it is

presumed that cultivated rice developed from wild species. The origin of cultivated rice is mainly based on the evidence of habitats where wild rice is grown and found (Grist, 1953).

The genus *Oryza* consists of species adapted to a broad range of habitats, from shady forests to vast stands in deepwater swamps. This occurred due to its long cultivation history and selection under different climatic, edaphic and environmental conditions. Wild rice can be found in the Himalayan foothills, Asian river deltas, and tropical Caribbean islands, the Amazon basin, and the inland swamplands of Southern, Western Africa and in the arid Savannahs of the tropics (Vaughan, 1994). Moreover, cultivated rice is grown as far as 50° N in China and 40° S in Argentina. This shows that rice can be grown at extreme latitude if the water supply can be adequately maintained (Yoshida, 1981).

The genus *Oryza* belongs to the tribe *Oryzeae*, which consists of 12 genera (Table 1.2), under the grass family of Poaceae (Gramineae). This genus consists of two cultivated species namely: *Oryza sativa* L. and *Oryza glaberrima* Steud., and about 20 related wild species (Table 1.3) (Vaughan, 1994). Both cultivated species are diploid. The common cultivated species *O. sativa* is mainly grown in Asia and is distributed throughout the tropics and in parts of the temperate regions of the world. On the other hand, *O. glaberrima* is endemic to West Africa and is distributed mainly in the Savannah, along the Southern fringe of the Sahara desert.

As mentioned above, two cultivated species of rice are grown world-wide; they have distinct characteristics and are evolved from different wild progenitors. The wild progenitor of *O. sativa* is very common in Asia. It is called *O. rufipogon,* and shows a range of variation, from perennial to annual types. The centre of domestication of *O. sativa* is somewhere in the piedmont zone of Assam, Upper Myanmar and Thailand, Southwest China and North Vietnam (Mannion, 1995) and between the Southern foothills of the Himalayas and Indochina. Its wild progenitor grows in these areas. The species *O. sativa* and its wild relative *O. rufipogon* are distributed throughout Asia, Australia and South America.

The wild progenitor of *O. glaberrima* (or African rice) is *O. barthii* (syn. *O. breviligulata*), an annual grass endemic to West Africa and thought to be originated in the Savannah zone of West Africa and Tanzania (Oka, 1991). The distribution of the wild rice types of *O. rufipogon* and *O. barthii* suggest different centres of domestication of cultivated rice. The geographical distribution of these species reveals that they evolved independently of their respective progenitors. They have discrete differences in terms of key characteristics, such as morphology; habitat and ecotypes (Oka, 1991). The main features of *O. glaberrima* are shorter ligules, less secondary branches of panicle and a thicker panicle axis compared to *O. sativa*.

Unlike *O. glaberrima*, the genus *O. sativa* L. has three sub species, viz. *indica*, *japonica* and *javanica*. The *indica* and *japonica* cultivars differ in many characteristics. The *indica* type is predominantly grown in humid tropical monsoon regions of South and Southeast Asia, and South and Central parts of China. The *indica* group consists of mostly long-grain slender and non-glutinous varieties, which tend to resist over cooking. The emphasis has been given on its improvement using both conventional and biotechnological methods (Khush, 1984; Toenniessen, 1990) because a majority of rice eating people consumes the *indica* type as a staple food.

The *japonica* varieties are grown in a wide range of latitudes. They are divisible into tropical and temperate subgroups and show different morphological and physiological traits from *indica* type. They are mainly grown in Japan, Korea and Northern China and in the former USSR (Chang, 1976). The *japonica* rice varieties have shorter, round and more glutinous grain as compared to the *indica* type. They often tiller freely and are reputed to respond to heavy fertilizer and have shorter; stronger stalks compared to the *indica* type. Finally, *javanica* rice is grown in the equatorial climates of Java and Indonesia and they are intermediate between the *indica* and *japonica* types, having long and bold type of grains.

Thousands of cultivars have evolved on the basis of their adoptions to local environmental conditions and this continuous process has resulted in a great number

of germplasm, which now exist in the world. The International Rice Germplasm Centre (IRGC) has registered 80,645 accessions (IRRI, 1995), and different national genebanks also registered a lot of accessions, such as, the Bangladesh Rice Research Institute, which has recorded over 7000 accessions (BRRI, 1997). These include breeding lines, land races, old cultivars and wild species of rice. This vast amount of germplasm are the potential sources of different biotic and abiotic stresses resistant genes, including the drought resistance types, which could be used for crop improvement.

Table 1.2 Genera, number of species, distribution, chromosome number, and spikelet structure in the tribe *Oryzeae*

Genus	Species (number)	Distribution	Chromosome number (2n)	Spikelet structure
Oryza	22	Pantropical(T)[a]	24, 48	Bisexual
Leersia	17	World-wide (t+T)	24, 48, 60, 96	Bisexual
Chikusichloa	3	China, Japan (t)	24	Bisexual
Hygroryza	1	Asia (t+T)	24	Bisexual
Porteresia	1	South Asia (T)	48	Bisexual
Zizania	3	Europe, Asia, North America (t+T)	30, 34	Unisexual
Luziola	11	North and South America (t+T)	24	Unisexual
Zizaniopsis	5	North and South America (t+T)	24	Unisexual
Rhynchoryza	1	South America (t)	24	Bisexual
Maltebrunia[b]	5	Tropical and Southern Africa (T)	Unknown	Bisexual
Prosphytochloa[b]	1	Southern Africa (t)	Unknown	Bisexual
Potamophila[b]	1	Australia (t+T)	24	Unisexual and bisexual

[a]T = tropical, t = temperate. [b] Considered as *Prosphytochloa* and *Maltebrunia* within the generic limits of *Potamophila*. (Adapted from Vaughan, 1994 and IRRI, 1995)

Table 1.3 *Oryza* species, major synonymy, chromosome number, genome group and potential traits

Species Complex/Species	Chromosome number	Genome group	Distribution	Potential or Useful traits
***Oryza sativa* complex**				
O. sativa	24	AA	World-wide	Cultigen
O. nivara	24	AA	Tropical and subtropical Asia	Partial resistance to stem rot
O. rufipogon	24	AA	Tropical and subtropical Asia	Tolerance to sheath rot
O. glaberrima	24	AA	Africa (mainly west)	Cultigen
O. barthii	24	AA	Africa	Resistance to green leaf hopper
O. longistaminata	24	AA	Africa	Bacterial blight resistance
O. meridionalis	24	AA	Tropical Australia	Drought avoidance
***O. officinalis* complex**				
O. officinalis	24	CC	Tropical and subtropical Asia	Thrips resistance
O. minuta	48	BBCC	The Philippines	Sheath blight resistance
O. rhizomatis	24	CC	Sri Lanka	Rhizomatous
O. eichingeri	24	CC	Sri Lanka, Africa	Not infected by yellow mottle
O. puntata	24, 48	BB, BBCC	Africa	Zigzag leaf hopper resistance
O. latifolia	48	CCDD	Latin America	Brown plant hopper resistance
O. alta	48	CCDD	Latin America	Striped stem borer resistance
O. grandiglumis	48	CCDD	South America	Large plant type
O. australiensis	24	EE	Australia	Rhizomatous
Ridleyanae Tateoka				
O. brachyantha	24	FF	Africa	Whorl maggot resistance
O. schlechteri	48	Unknown	Papua New guinea	Stoloniferous
***O. ridleyi* complex**				
O. ridleyi	48	Unknown	Southeast Asia	Stem borer resistance
O. longiglumis	48	Unknown	Irian Jaya, Indonesia	Shade tolerance
Granulata Rochev.				
***O. meyeriana* complex**				
O. meyeriana	24	Unknown	Southeast Asia	Shade tolerance
O. granulata	24	Unknown	South and Southeast Asia	Shade tolerance

(Adapted from Vaughan, 1994 and IRRI, 1995)

1.3 Constraints for rice production in its ecosystems

The term ecosystem is defined by Arthur Tansley as a unit comprising a group of organisms and their environment (Mannion, 1995). The rice ecosystems are rainfed lowland, upland, flood-prone land or deepwater, and irrigated ecosystems. They are mainly dependent on water regime, rainfall pattern, depth and duration of flooding and drainage. More than 50% of the world's rice crop area is under irrigation and contributes to about 75% of the world rice production. In an irrigated ecosystem, rice productivity is high, and this is largely due to the adoption of modern rice technology by farmers. Upland ecosystems account for about 15% of the world rice production area but contribute only 5% of total rice production. Deepwater ecosystems account for about 10% of the world's rice crop area and contribution to total production is also around 5%. No technological breakthroughs have been achieved in the upland and deepwater ecosystems, (David, 1991), and as a result, rice production is always subject to drought, flood and saline conditions.

Rainfed lowland ecosystems predominate in the world's most densely populated rural regions, such as South and Southeast Asia. They contribute about 17% of the world's total rice production, from about 25% of the world's total rice land, which means that productivity is low in rainfed ecosystems compared to irrigated ones. This low productivity is due to the fact that floods or drought or both occur almost every year. Moreover, salinity causes both genetical and physiological complex changes in the expression of plant genes (Flowers *et al.*, 2000; Yeo *et al.*, 2000; Koyama *et al.*, 2001). This also causes low productivity in rainfed lowland ecosystems. It is clear from the above statistics that the production of rice per unit area is less in rainfed lowland, upland, and flood-prone or deepwater ecosystems compared to irrigated ecosystems. One reason may be the lack of appropriate varieties for these ecosystems. Therefore, rice production could be increased more efficiently in these ecosystems through the selection or development of appropriate varieties, as the production is poor or indigent.

The interactions of soil, water, climate and the plant are the key elements in the production of cereal grains. Major environmental stresses to which plants are generally subjected are drought, high temperature, low temperature, flood, salinity, and high radiation (Boyer, 1982; Herdt, 1991) and account for about 85% of the total crop plants production losses (Boyer, 1982). Among these, drought and high temperature are considered as major factors, which limit the yield in crop plants (Boyer, 1982). Water is the most important factor than any other for the development of field crops, because severe water stress arrests activities for growth and development of crops. Moreover, environmental concern regarding the adverse effects of irrigation and flood control makes for difficult choices as regards the use of fresh water for food production.

In Asia, rice is grown on small family-based farms, an average farm size being 1 ha or less (David, 1991). It accounts for 30-50% of total income. As a result, rice is regarded as a strategic commodity and an important component of culture in many Asian countries. As mentioned earlier, rice production should increase by at least 50% from present production levels, given the decrease in present cultivable land resources. So there is a need to find alternative ways to increase production. About 200 million hectares of rainfed lowland in Africa and Latin America are not cropped and are under-utilised due to lack of appropriate technology. New technologies and varieties that are suitable for upland and rainfed lowland rice areas could bring much of that land into production.

Drought limits the yield at some stages of the life cycle in rainfed and upland conditions. In uplands and rainfed lowland areas, farmers generally do not have access to irrigation, resulting in low yield due to water stress caused by uneven distribution, and unpredictable and insufficient rainfall patterns (Chandra-Babu *et al.*, 1996; Hossain, 1996). More than one-third of the South and Southeast Asian rice lands are under rainfed lowland conditions, where drought and flood along with salinity hamper crop production at some stages of crop growth. In Eastern India, farmers identified drought stress as the foremost constraints to higher yield in upland and rainfed ecosystems (Herdt, 1996). These ecosystems may experience frequent

and severe water stress during the rice-growing period at any time. The world's rainfed lowlands as well as uplands offer tremendous potential for increasing rice production and for making rice profitable (IRRI, 1982a). Therefore, it seems that productivity could be increased in upland and rainfed ecosystems with the development of drought resistant varieties.

As mentioned above, upland and rainfed lowland are the two major ecosystems where water stress or drought is likely to occur (Toenniessen, 1991; Mackill, 1996). Ribaut *et al.*, (1996) described drought as an important climatic phenomenon, which after soil fertility, ranks as the most severe limiting factor for maize production in developing countries. In Asia, 30-40% of rice lands is subject to monsoon flooding. Monsoon rainfalls fluctuate drastically, from heavy to minimal downpours. Therefore plant breeders need to develop varieties for both drought resistance and submergence tolerance to increase productivity. This has become a major challenge to them. Plant response to drought is a complex phenomenon and different drought resistance mechanisms might act together to minimize yield losses under a given environmental stress. From the above discussion it is apparent that there is a need for development of varieties, which are suitable for drought or water limiting conditions.

1.4 Genetic improvement of the crop

The strategies for crop improvement include identification and selection of the desired traits, which contribute to the improvement of the performance of crops including drought conditions. The trait-based crop improvement strategy involves selective accumulation of the traits that contribute to drought resistance for a specific environment (Garrity *et al.*, 1982; Blum, 1983; Rosenow *et al.*, 1983; Bidinger *et al.*, 1992). The most important requirement for drought resistance is correct phenological development, which matches crop development to the pattern of water availability for all drought environments. The identification and selection of correct phenological genotypes is important, along with development plasticity, a mechanism where the duration of the growth period varies with the availability of water. Phenological plasticity is considered as an important characteristic in

drought prone environments. The identification and selection of genotypes for desired traits in the field is sometimes difficult due to the large environmental influence on the expression of the traits, the cost and labour involved in conducting such multi-location trails and also the lack of good control over stress treatments. Different novel methods have facilitated the above-mentioned strategies. However, the establishment of physio-morphological characteristics is the first step for the description, classification and identification of crop germplasm.

Generally, *japonica* rice has thicker and longer root systems compared to *indica* and *japonica* is often grown as an upland crop. The wide range of root development systems also indicated the adaptability to upland conditions (Oka, 1991). So drought resistance traits or genes may be available in *japonica* rice. The varieties of *O. glaberrima* have some genes that are tolerant to adverse biotic and abiotic conditions; thus drought resistance genes might be available from the species. Moreover, the wild relative of rice contains numerous useful genes including drought resistance ones. This means that drought resistant varieties can be developed if the above-mentioned germplasms can be exploited.

However, several reports have shown that hybrid sterility and hybrid breakdown are common in crosses within and between cultivated species of rice and these are genetically complicated (Lynch *et al.*, 1991; Oka, 1991; IRRI, 1996). Moreover, a range of variation in the rate of success is also found even in crosses between the same genepool with shared common forms. Crosses between *indica* and *japonica* always show reduced fertility. The hybrids of crosses between *O. sativa* and *O. glaberrima* are sterile (Oka, 1991). These types of abnormalities occur not only in the crosses between *O. sativa* and *O. glaberrima*, but are also observed in other crosses, such as crosses between *O. sativa* and *O. rufipogon* (IRRI, 1996). The interspecific hybrids show abnormalities in spindle formation and non-synchronisation of chromosome movement, resulting in the formation of restitution nuclei carrying an unreduced set of chromosomes. It can be summarised that in *Oryza* species, F_1 sterility and other dysfunctions occur irrespective of

genetic distance. If these barriers can be overcome, desirable genes from different sources could easily be transferred and incorporated into cultivated varieties.

As mentioned earlier, drought resistance traits are available in wild rice (Table 1.3) and in some distantly related species, such as maize, sorghum and wheat. The rate of success for transferring genes from wild relatives to cultivated rice is very low and it is less than 1% (Oka, 1991). This implies conventional plant breeding should be supplemented by other technology because new strategies are continually sought to improve the efficiency of plant breeding programmes. Genetic transformation methods could be used for transferring specific gene(s) if the traits are identified. However, this technique is very costly and feasibility in many poor developing countries is questionable. Therefore there is a clear need for other technology for the improvement of crop; the implication is that improved technology can increase crop productivity. Therefore a preliminary attempt has been made to develop a technique for gene transfer through isolation of sperm and egg cells and fusion of the isolated gametes by using *indica* type *O. sativa* species.

It is apparent that drought resistant varieties are needed for rice if the production in rainfed and upland ecosystems is to be extended. Therefore, the overall objective of this study is to develop suitable technique(s) in order to improve rice productivity in drought stress areas. The specific aims of the project are as follows:

1. To identify accessions with potential drought resistance genes through physio-morphological and isozyme systems studies.
2. To transfer drought resistance genes to existing high yielding lines or varieties through the *in vitro* fertilization.

CHAPTER TWO

Review of literature

2.1 Assessment of drought resistant rice

In general, there is no accepted definition of drought resistance, and the phenomenon is perceived in various ways in different places. It has specific effects according to location, land use and climate. Indeed, the term is somewhat ambiguous and difficult to interpret as different authors have explained it in different ways. Use of soil for quantification of drought stress rather than plant water status is an added complexity to understand (Sullivan, 1971).

According to precipitation criteria, drought is defined as the shortage of water in a period of time in a particular agroceosystem (Nix, 1982). This implies that the primary reason for drought is lack of precipitation. It is well known that the water from precipitation moves in different ways, through evaporation, infiltration, runoff, drainage, soil conservation and water uptake by plants. These factors are highly interrelated and occur simultaneously. Temperature can be influential often in various ways. As a result, temperature is also included as a drought parameter, specially given the importance of evaporation. Thornthwaite in 1948 suggested a more realistic basis for concepts of water balance involving the recording of water transfer into six categories, *viz.*, precipitation, potential evaporation, actual evapotranspiration, soil moisture storage, soil moisture deficit, and soil moisture surplus (Nix, 1982).

Some authors have classified drought into two types namely: meteorological and agricultural depending on the data from meteorology and agro-ecosystems (Kramer, 1980; Swindale and Bidinger, 1981). Meteorological drought could be defined as the percentage of rainfall over a period of time, which is below the long-term mean rainfall of that area. Agricultural drought, on the other hand, could be defined as the unavailability of sufficient water for a crop during its growing period. Agricultural drought may include some different threshold conditions and criteria, which may be

related to specific crop plants (IRRI, 1982). This makes breeding programmes for drought resistance difficult, and progress is slow because the different types of drought may require different approaches for breeding.

2.1.1 Mechanisms of drought resistance

Drought is the most important abiotic stress limiting the production of rice (Toenniessen, 1991) and maize (Ribaut *et al.*, 1996). Nguyen and Joshi (1994) reported that plants respond to different kinds of stress depending upon their genetic make-up. Understanding of the physiological, molecular and biological effects of these stresses constitutes the first step towards the development of strategies for designing stress resistant genotypes. The major physiological components of drought resistance in rice are the root system, osmotic adjustment and cuticular transpiration (Nguyen *et al.*, 1997).

There are two main strategies in drought resistance, namely: avoidance and tolerance. The dehydration avoidance mechanism helps a plant to maintain cell turgor and cell volume resultant of high leaf water potential, which helps to avoid the stress experienced by the plant tissues. The avoidance involves enhancement of water uptake by deeper and more extensive root systems, or by reducing water loss by stomatal closure, leaf movement, and low epidermal conductance. These involve osmotic adjustment for turgor maintenance, allowing stomata opening, photosynthesis and leaf expansion over water stress (Steponkus *et al.*, 1980; Collinson *et al.*, 1997; Azam-Ali and Squire, 2002). Drought tolerance mechanisms can help a plant to maintain important metabolism or physiological functions at lower leaf water potential because its tissues are able to tolerate dehydration using superior protoplasmic tolerance of desiccation.

The other strategies for drought resistance are escape and recovery. A plant can escape drought by completing its life cycle before the water stress occurs and this is mainly observed in desert ephemerals. Desert ephemerals have a special phenology with photoperiod insensitivity and developmental plasticity, which allows them to

flower at any stage of plant development subject to water availability. The early maturity of upland rice and the photoperiod sensitivity of lowland rice also constitute escape mechanisms. Drought recovery lies in the budding capacity of the plant. When drought occurs at an early stage of crop development, some genotypes are able to produce more tillers upon relief of drought, and these tillers are productive if the remaining growing season is long enough to complete grain filling (Fukai and Cooper, 1995).

Most of the drought resistance traits of rice represent avoidance mechanisms, such as deeper and denser root systems and stomatal control (Mackill, 1996). O'Toole (1982) classifies the drought resistance mechanisms of rice into root-related traits, shoot-related traits, and reproductive stage specific traits. Root-related traits are deeper and denser root systems, high root penetration ability through soil hardpan, and root osmotic adjustment. Root depth is the plant trait most strongly related to drought avoidance in upland rice culture (Chang *et al.*, 1987).

Different authors have reported that the most important root-related traits for combating drought by water extraction from the soil are root number, root thickness, root depth and branching, root length density, and root penetration ability through compacted soil layers (O'Toole, 1982; Yoshida and Hasegawa, 1982; Thanh *et al.*, 1999). Root thickness can be used as a selection index for xylem size up to about 1.2 mm root diameters in rice (Yambao *et al.*, 1992). Different root characteristics like root penetration ability are quite difficult to measure directly and accurately in the field due to lack of uniform soil compaction, and it is also an intensively laborious task. In rainfed lowland ecosystems impenetrable plough pans (hardpan) are formed due to puddling operations during pre-transplanting. During drought, rice roots cannot penetrate to the deeper soil (due to hardpan) to extract water from the deeper soil layer. Drought also increases mechanical obstruction (Thangaraj *et al.*, 1990). Under the above conditions, the productivity of crops could be maintained if cultivars had some water stress resistance mechanism.

Shoot-related traits are stomatal characteristics, leaf rolling, a thicker epicutical wax layer, and osmotic adjustment. Matthews *et al.* (1990) described that drought resistance sorghum lines showed more leaf-rolling than the susceptible lines, reducing the effective area of the uppermost leaves by about 75%. Leaf-rolling in the resistant lines occurred over a very narrow range of leaf water potential (-2.0 to -2.2 mega Pascal-MPa) compared to susceptible lines. The strong correlation between the light-extinction coefficient (k) and leaf-rolling indicated that changes in k over the season were in part due to the leaf rolling abilities of the lines. Lines exhibiting leaf-rolling had higher leaf conductance, although transpiration rate per unit leaf area was similar. Leaf-rolling may alter the leaf surface micro climate so that stomata may remain open and growth continue without association of high rates of water loss.

Reproductive stage specific traits are early morning anthesis, increased panicle diffusive resistance, tolerance for high spikelet temperature, demobilisation and translocation of stored water.

2.1.2 Leaf water potential

Variation in leaf water potential among cultivars or strains under moisture stress conditions were found rice (O'Toole and Moya, 1978; O'Toole and Cruz, 1979). This was also reported in wheat (Fischer and Sanchez, 1979; Quarrie and Jones, 1979; Blum, 1980), sorghum (Blum, 1974; Ackerson *et al.*, 1980) and bambara groundnut (Collinson *et al.*, 1997; Azam-Ali *et al.*, 2001). It has been reported by Gomathinayagam *et al* (1988) that leaf water potential and leaf area were the most important parameters for drought resistance in rice. A difference in leaf water potential of up to 1.0-1.3 MPa between genotypes was found in rice (O'Toole and Moya, 1978) and wheat (Blum, 1980).

In rice, midday leaf water potential falls, even in irrigated conditions, because of a lag between water absorption by roots and transpiration through the leaves. This indicates that midday leaf water potential may be a criterion for selecting a drought resistant plant. Bashar *et al.* (1990) conducted an experiment on midday leaf water

potential in rice and suggested that leaf water potential may be used as a selection criteria for drought resistance.

2.1.3 Osmotic adjustment

Osmotic adjustment, a process of active solute accumulation within cells under drought stress, is receiving increasing attention as a probable component of drought resistance in crop plants (Turner *et al.*, 1986a; Ludlow and Muchow, 1990; Turner *et al.*, 2001; Azam-Ali and Squire, 2002). This process occurs in all tissues of the plant and appears to be expressed differentially among different species and within different genotypes of the same species (Ludlow and Muchow, 1990, Ludlow *et al.*, 1994). It is a multifaceted trait and has potential benefits for plants in water stress conditions. It promotes leaf survival or to stay green by increasing both avoidance (enhancing root growth and extraction of soil water) and tolerance of dehydration. A phenotypic correlation between grain yield and osmotic adjustment has been shown in wheat (Morgan *et al.*, 1986) and *Brassica* (Singh, 1989). Osmotic adjustment helps to maintain the turgor of both shoots and roots at the time of water deficit (Turner and Jones, 1980). Osmotic adjustment is not an inherited trait, but the capacity for osmotic adjustment is inherited. It is an inducible trait that occurs only when water stress develops and turgor was fully maintained in stem and pulvinus under drought conditions (Virgona and Barlow, 1991).

Significant genetic variation in osmotic adjustment has been observed in different crops, including rice, subjected to water stress (Hsiao *et al.*, 1984; Turner *et al.*, 1986a; Lilley *et al.*, 1996). Blum (1989) reported that barley (*Hordeum vulgare* L.) genotypes differed significantly in osmotic adjustment, which ranged from -0.17 to 0.46 mega Pascal (MPa) when the water potential was the same. Morgan (1983) found that wheat (*Triticum aestivum* L.) genotypes with a greater capacity for osmotic adjustment gave more yield under drought conditions. Grumet *et al.* (1987) selected barley isopopulations differing in constitutive osmotic adjustment by about 0.1 MPa. Dehydration avoidance was enhanced when osmotic adjustment promoted

root growth. Osmotic adjustment is also effective in plant tolerance to salinity and freezing stresses, both of which involve a component of water deficit (Blum, 1988). It has been shown that growth and yield under water-limiting conditions can be improved by selecting lines with higher levels of osmotic adjustment in wheat (Morgan, 1983), sorghum (Ludlow *et al.*, 1990), barley (Blum, 1989) and grain legumes (Turner *et al.*, 2001). It is reported by Turner *et al.* (1986a) that osmotic adjustment occurs in rice subjected to water deficit, and a genetic variability is also observed in osmotic adjustment among rice lines.

2.1.4 Root penetration

It is generally assumed that an essential feature of a drought resistant plant is a deep, wide spreading and branched root systems, such as that of sorghum (Kramer, 1969). Extraction of moisture by the deeper roots helps the plant to maintain turgidity and growth under mild drought or enables them to survive under severe drought stress. Root growth is influenced by genotype and the physical characteristics of the soil, such as soil hydrology and soil mechanical impedance.

Root growth and extension of plants decrease with increase in soil strength. A soil strength greater than 0.5 MPa and a soil bulk density greater than 1.5-g/cm^3 hamper root growth and penetration below 10-15 cm of depth from the soil surface (Hasegawa *et al.*, 1985; Thangaraj *et al.*, 1990). The presence of compacted soil layers act as physical and physiological constraints to overall plant growth (Tu and Tan, 1991) by preventing the downward growth and distribution of the plant root systems (Yu *et al.*, 1995). Compacted soil layers reduce leaf area, dry matter accumulation, root elongation rate, transpiration rate, and crop yields (Pathan, 1998). Yield was increased in cotton and soybean by mechanical disruption of the compacted soil layers. However, mechanical disruption is expensive and requires reiteration after a few years (Busscher *et al.*, 1986). Yu *et al.* (1995) also reported that the ability of roots to penetrate compacted soils can be beneficial, as this will avoid drought stress.

The differential response of rice genotypes to soil and atmospheric water stress has been treated as a root system characteristic (Armenta-Soto *et al.*, 1983; Chang *et al.*, 1972). A significant genotypic variation exists for root penetration ability among different rice cultivars (Yu *et al.*, 1995). The *indica* and *japonica* subspecies of rice are usually adapted to lowland and upland ecosystems, respectively. They differ generally in their root morphology and rooting patterns. Cultivars of upland origin are more deeply rooted and have a larger diameter of the main root axes as compared to cultivars of lowland origin (Yoshida and Hasegawa, 1982; Price *et al.*, 1997). Root morphology and rooting patterns directly affect the amount and timing of water availability to a crop (Champoux *et al.*, 1995). Several authors have reported that thick and long rice root systems help the root to penetrate the compacted soil layer for better availability of ground water (Yoshida and Hasegawa, 1982; Ekanayake *et al.*, 1985 and1985a; Thanh *et al.*, 1999). A well developed long thick root has a positive effect on the yield of upland rice under water stress conditions (Yadav *et al.*, 1997) and better root growth is also associated with drought avoidance (Price and Tomos, 1997). Thick roots produce more and longer branched roots that increase root length density and increase water uptake capacity (Ingram *et al.*, 1994). Thick roots are known to have a wider xylem diameter and consequently less axial resistance to water flow along the xylem. Both axial and radial resistance limit water movement (Yambao *et al.*, 1992).

Ludlow and Muchow (1990) and Turner *et al.* (2001) suggested that a deep and vigorous root system could increase water transpiration, allowing the plant to avoid water deficits at critical growth stages and to contribute to a higher yield in water limited environments. The ability to maintain water uptake during drought appears to be a major attributer conferring increased drought resistance to traditional rice varieties, while increased rooting depth and density would improve the capacity to extract available water (Cruz *et al.*, 1986; Fukai and Cooper, 1995). The configuration of root systems under field conditions is largely determined by factors such as fertility gradients, soil moisture, mechanical impedance and aeration (Lynch, 1995), which are in turn affected by soil climate and cropping patterns and systems.

2.2 Yield response to soil moisture status

There is no doubt that the timing and duration of water stress has a major effect on crop yield, because drought does not need to be prolonged to affect crop yield. Even one day water stress can have some effect on yield if the stress is severe (Garrity and O'Toole, 1994; Garrity and O'Toole, 1995). Ingram and Yambao (1988) reported that water deficit during the vegetative stage had no effect on the grain yield of rice. However, during the reproductive phase, water deficit over 5 or 10 days reduced yields by 25 to 45% and a water deficit over 15 days reduced yield by up to 88%. These results indicate that rice yield is reduced most when drought occurs during the reproductive stage. However, some cultivars have some capacities to maintain green leaves longer period resulting in increase dry matter production and are believed to be drought resistant (Jearakongman *et al.*, 1995). Howell and Hiller (1975) reported that sorghum had the highest yield reduction when water deficit occurred at the booting stage of growth. Similarly, Hanks *et al.* (1978) carried out experiments on corn using different levels of irrigation, revealing the importance of irrigation during the vegetative stage for yield maximisation. Winter maize reached a maximum value of 6t/ha when irrigated at 0.055 MPa soil moisture tension during the vegetative stage and at 0.033 MPa during the reproductive stage (Jana and Puste, 1985). Similar results were also reported for maize and sunflower (FAO, 1979).

Maximum yield is achieved by irrigating at a particular matric potential. Taylor (1965) listed all the matrix potentials at which to irrigate crops in order to obtain maximum yield (El-Husseini, 1993). Singh and Malik (1983) conducted experiments on dwarf wheat, recording a maximum grain yield of 5.2 t/ha under no water stress. When a severe water stress of -1.5 MPa was imposed, yield reduction of 33.9% occurred. Hedge and Srinivas (1989) reported that banana fruit yield increased by scheduling irrigation at a soil matric potential of –0.025 MPa. Banana is sensitive to soil moisture stress; hence, growth and yields were adversely affected by water deficits especially when a soil matric potential below –0.05 MPa (FAO, 1979).

2.3 Breeding strategies for rice improvement

Rice is a self-pollinated species and the systematic breeding probably started at the end of the 19th century. Since then various methods have been applied from conventional hybridisation to novel breeding methods, such as transformation, protoplast regeneration and fusion for rice crop improvement and some of the approaches are described below.

2.3.1 Conventional approaches

The most common method for rice breeding is hybridization and initially, rice was bred so far for agronomically important traits, such as disease, insect resistance and salt tolerance. Nowadays selection is used mainly to obtain desired genotypes from segregating populations after hybridization (Poehlman and Sleper, 1995). Many new cultivars have been developed through this method. Rice production increased significantly after the establishment of the International Rice Research Institute (IRRI) and after the development of semi-dwarf rice varieties, as well as for other technological developments by the IRRI. Since then, different national agricultural research organizations have launched the "*Green Revolution*" programme in rice. This has led to a major increase in rice production in many rice growing countries. Nowadays most of the rice growing countries in Asia have become self-sufficient in food production but still need to increase production due to population increase.

The genetic variability in traits, such as tolerance or resistance to drought, salinity and temperature is difficult to obtain because of the nature of the cultivated rice germplasm. Wild relatives of rice on the other hand have potential sources of various traits including the above mentioned (Table 1.3). Hence wild relatives of rice can play an important role for rice improvement. Rice scientists have been trying to incorporate these useful genes into desired elite cultivars for a long time. However, this is not an easy job, as many barriers are encountered in transferring these useful genes from wild species to cultivated rice (Khush and Brar, 1992). Crossing barrier is the most common problem, which is due to disharmonies of pollen pistil interaction,

chromosomal elimination or genomic imbalance and these have resulted in early embryo degeneration. However, some success has been achieved by different scientists to transfer genes, such as the grassy stunt virus resistance gene from *O. nivara* (Khush, 1977), the cytoplasmic male sterility gene from *O. perinis*, the brown plant hopper (BPH) resistance gene from *O. officinalis*, BPH and bacterial leaf blight resistance genes from *O. australensis*, and the blast and bacterial leaf blight resistance genes from *O. munata* (IRRI, 1991). Few reports have been found so far on transferring drought resistant traits, although there is a scope to improve rice by incorporating useful genes, such as drought resistant traits from wild rice to cultivated rice.

2.3.2 Breeding for drought resistance

It is difficult to conduct a screening programme for root penetration ability in compacted soil layers (soil hardpan) due to lack of efficient, reliable techniques and the inability to create uniform compacted soil layers in the greenhouses and in fields. The available analytical techniques for evaluating root traits are tedious and time consuming (Robertson *et al.*, 1985; Ingram *et al.*, 1990). This puts breeding programmes in difficulty. Busscher *et al.* (1986) reported that a soil strength greater than 1 MPa reduces root growth and when it is greater than 2 MPa, it completely prevents root growth. Similar results were also reported by Kandasamy (1981) for artificially generated compacted soil layers with strength of 1 to 2 MPa. Yu *et al.* (1995) developed an efficient screening technique for the study of root penetration, using wax petrolatum layers to simulate compacted soils. The wax petrolatum layers consisted of 60% wax and 40% petrolatum white, with a resistance of 1.4 MPa at 27°C. They found that the dry land origin cultivars had greater root penetration ability than the cultivars of the wetland origin.

Genetic variability in root length, root tip thickness, root number, root weight and shoot weight have been recognized in rice (Armenta-Soto *et al.*, 1983, Yu *et al.*, 1995). Chang *et al.* (1982) reported that additive gene action was related to maximum root length, root thickness, root number, and root-shoot dry weight ratio. Plant height

and tiller number showed a combination of additive and dominance effects, while the root and shoot dry weights showed only dominant gene action. The root length was positively correlated with plant height, root and shoot dry weight, root-shoot ratio, and root thickness. Root thickness was positively correlated with plant height and root-shoot dry weight ratio. The root number was positively correlated with the tiller number and the dry weight of roots and shoots. It is also well documented that root growth varies across rice types and varieties.

Japonica rice varieties generally have thicker and longer roots compared to the *indica* type. *Indica* upland rice produces more long and thicker roots than lowland rice (Chang *et al.*, 1982; Ekanayake *et al.*, 1985). Therefore it can be assumed that drought resistant traits might be available in these genotypes, which are related to root penetration ability. There is scope to develop root related drought resistant cultivars by incorporating these traits. However, the hybrids of crosses between *indica* and *japonica* are sterile. Moreover, field screening for root traits is difficult, which in turn slows down the breeding progress. Once the traits for drought resistance have been identified, methods can be explored to incorporate these traits into elite varieties.

2.3.3 Use of genetic markers with special reference to isozyme

One of the most important advantages in the field of biotechnology is the utilisation of genetic markers. Generally, markers may be categorised as morphological or visible, protein and DNA markers. Although both morphological and protein markers are limited in number, they can also be used for identifying desirable traits. Molecular markers are discrete, codominant, non-deleterious characters that are not affected by environment and are free of epistatic interaction (Tanksley *et al.*, 1989; Paterson *et al.*, 1991).

Markers are generally used for genetic diversity analysis, germplasm evaluation, construction of genetic linkage maps, genome analysis, gene tagging, map based gene cloning and marker aided selection (Anand, 1998; Jung, 2000). Most of the

agronomically important traits are controlled by polygenes. Plant breeders can use genetic markers as a selection index, because genetic markers are associated with economically important plant traits (Paterson *et al.*, 1991; Neale *et al.*, 1992; Harris *et al.*, 1994). Superior alleles can be efficiently selected if molecular markers are closely linked to the allele or genomic region of interest. Selection based on molecular markers can overcome the barriers associated with low heritability and recessive and difficult screening assays.

Genetic variability or diversity has traditionally been determined through the phenotypic characteristics of a plant population because such traits are easy to observe and measure. Moreover, phenotypic diversity in most cases represents a major proportion of the genetic diversity in a population. Furthermore, some of the economically important physio-morphological traits such as taste or aroma in rice, and the maturity period are directly utilised to assess the potentiality of germplasm. However, the level of accuracy in determining the genetic variability or diversity solely through morphological characters is less because of the influence of environments on the phenotypic expression. This is especially important for agronomically important traits, such as yield and the quality of the grains, in other words, the quantitative traits. Therefore, the need for more reliable techniques is obvious. The recent development of DNA marker and protein marker techniques can efficiently supplement morphological or visible markers.

An isozyme is a biochemical marker, *i.e.* a protein or molecular marker which is also used to measure genetic variability in plant species (Baily, 1983; Haq, 1996a; Omara and Maria, 2000). It has been used in taxonomic, genetic, evolutionary and ecological studies. It has also been utilised for cultivars or line identification (Hamrick *et al.*, 1992). In spite of DNA markers, such as RFLP and RAPD, isozymes are still widely used, because of their simplicity and low cost in species delimitation and conservation (Chamberlain, 1998), assessment of genetic variability in species and populations, cultivar identification, gene flow and evolutionary studies (Gauthier *et al.*, 1998; Jung, 2000; Volis *et al.*, 2001). Moreover, in most cases, isozyme analysis can be done rapidly. So for rapid assessments of the variability of a population, the

isozyme probably represents the best approach. It has already been used in the classification of rice germplasm (Second, 1982; Glaszmann, 1985; Glaszmann, 1987; Noboru *et al.*, 1997).

2.3.4 Wide hybridization

The term hybridization is used for a wide range of processes involved in cross-pollination. Wide hybridization usually refers to gene flow between populations, which are reproductively isolated from one another and includes interspecific and inter generic crosses (Hadley and Openshaw, 1980). Wide hybridization is an important cytogenetic and plant breeding tool to improve crop plants. It allows the introduction of alien variations and transfer of desirable gene(s) from wild to cultivated species. It is well known that wild relatives of crop species are important sources of genetic variability for various economically important characteristics, for instance tolerance to biotic and abiotic stresses and male sterility, increased biomass, grains yield and improved quality characteristics. Several reports have been published in relation to the important role of wild relatives in crop improvement (Brar and Khush, 1986; Kalloo, 1992).

Transfer of genes from wild species to cultivated species is not straightforward and often encounters barriers for free gene(s) transfer. However, interspecific crosses are designed in breeding programmes to transfer genes or chromosome blocks, and several successes have been documented (Haq, 1996b). The successful utilisation of alien genetic material depends not only on effective wide crosses but also on subsequent inter-genomic recombination.

Internal barriers to genetic interchange between related populations occur as a result of either sexual incompatibility or hybrid breakdown. They usually occur when attempts are made to cross between different species or members of different genera. Sexual incompatibility, which prevents the fertilization of the egg cell and formation of the zygote due to disharmonies existing in the pollen pistil interaction, are considered as pre-fertilization barriers. Hybrid breakdowns due to failure of hybrid

embryo development, or hybrid plant inviability, weakness or sterility are referred to as post-fertilization barriers.

Several techniques have been designed to overcome the barriers for successful viable hybrid production between two incompatible species (Brar and Khush, 1986; Khush and Brar, 1992; Haq, 1996b). Some of these techniques are described below.

2.3.4.1 Embryo rescue method: Abortion in hybrid embryos is a very common feature of wide crosses. It occurs at different stages of development, depending on the genomic relationships of the two parents. Such abortic embryos can be dissected from the developing ovaries or ovules, and cultured in a nutrient medium. In some cases, it is not possible to excise embryos from ovules, and in that case whole ovules or ovaries are cultured for embryo rescue. Using embryo rescue, a large number of interspecific and intergeneric hybrids have been developed in different species including *Oryza* (Brar and Khush, 1986; Khush and Brar, 1992; Sharma *et al.*, 1996). The success of the embryo rescue method depends on the medium composition and the developmental stage of the embryo.

2.3.4.2. Amphidiploids: Hybrids of crosses between distantly related species often show high levels of sterility. This sterility may be due to genic or chromosomal differences between the two parents. Such sterility can be overcome by doubling the chromosome number of the hybrids in order to produce amphidiploids.

2.3.4.3 Bridging species technique: Sometimes direct crosses between species with the same or different ploidy levels are difficult or impossible to achieve. A third species (bridge species) is used to produce successful crosses. This technique has been used to make wide crosses in several species such as wheat (*Triticum*), potato (*Solanum*) and tobacco (*Nicotiana*).

2.3.4.4 In vitro fertilization: Any manipulation of excised maternal and paternal tissue to accomplish pollen tube penetration to the embryo sac for fertilization is

referred to as *in vitro* fertilization. It is an important method to overcome both pre- and post-fertilization barriers.

2.3.4.5 Use of growth hormones: Various growth hormones have been used to stimulate pollen tube growth and embryo development (Khush and Brar, 1992). Growth hormones prolong the receptivity of stigma and prevent early abscission of pollinated flowers. Chances when producing wide hybrids can be improved considerably using gibberellic acid (GA_3) and other growth hormones.

2.3.5 Somatic hybridization

Somatic hybridization is the *in vitro* fusion of plant protoplasts derived from somatic cells of plants with different genetic structure. The isolated protoplasts, as naked cells, tend to fuse themselves, and can be induced to fuse with other protoplasts of different species, thereby providing opportunities for the creation of novel plant hybrids. This technique offers unique advantages over sexual hybridization in terms of combining unrelated genomes and cytoplasms. This method can be used to transfer novel genetic traits to overcome strong sexual incompatibility barriers when conventional hybridization is not possible (Kumar and Cocking, 1987; Tang *et al.*, 2000). Moreover, it can be used in genetic transformation by incorporation and expression of foreign DNA, usually in plasmid form. However, such techniques are always linked to the subsequent recovery of whole plants *i.e.* plant regeneration, which is often associated with problems.

Cocking (1960) published a pioneer report on the enzymatic isolation of higher plant protoplasts. Since then, attempts have been made to regenerate rice protoplast from their fused products (protoplast fusion). These have been aimed at transferring novel genetic traits across sexual incompatibility barriers, and introducing cytoplasmic male sterility into the desired rice cultivars (Kumar and Cocking, 1987). The initial success on rice protoplast isolation and culture were based on the *japonica* varieties and was first reported by Fujimura *et al.*, (1985) and Yamada *et al.*, (1986). However,

it is difficult to regenerate protoplasts of *indica* compared to *japonica* rice. Lee *et al.* (1989) developed a protocol for regeneration of *indica* rice from protoplasts.

Abdullah *et al.* (1986) reported a rapid, efficient and reproducible method for fertile plant regeneration through somatic embryogenesis from cell suspension-derived protoplasts of two *japonica* cultivars (Taipei 309 and Fujisaka 5). After that, several successful reports on protoplast culture with regeneration of *japonica* (Thompson *et al.*, 1986; Jenes and Pauk, 1989; Li and Murai, 1990; Jain *et al.*, 1995) and *indica* rice have been published (Datta *et al.*, 1990; Gupta and Gupta, 1995, Jain *et al.*, 1995). Successful reports have also been published in *javanica* rice (Suh *et al.*, 1992; Tang *et al.*, 2000). However, no successful report has been published in *O. glabarrima* for protoplast isolation and culture so far. Considering the problems associated with the success of *indica* varieties, further research is needed to overcome the problems.

Successful protoplast culture and plant regeneration systems permit selection of somaclonal variations among protocloned plants. Generally, plants regenerated from protoplasts exhibit more variation than plants regenerated from explants due to the cytological instability associated with the protoplast isolation process (Karp, 1991). Among protoplast-derived rice plants, variation was also found in ploidy level (Gurderdoni and Chair, 1992; Mezencev *et al.*, 1995).

The main areas of application of protoplast technology in rice improvement are genetic transformation, using direct DNA uptake by protoplasts, the transfer of cytoplasmic male sterility, disease and insect resistance and tolerance for abiotic stress from wild rice through protoplast fusion. The application of protoplast technology in rice crop improvement programmes is limited by the selective response to genotypes and poor reproducibility (Lynch *et al.*, 1991; Hodges *et al.*, 1991).

2.4 *In vitro* fertilization

The difficulties that occur during wide hybridization can be overcome by other techniques, such as isolation and manipulation of male and female gametes in their fusion. The isolation of gametes and their *in vitro* fusion techniques have been developed for some species, such as *Zea* (Kranz *et al.*, 1991a&b) and *Nicotiana* (Tian and Russell, 1997; Tian and Russell, 1997a). These techniques seem to be logical and appropriate to by-pass the difficulties, which occur during crosses between inter-specific and between *indica japonica* subspecies. No report has been published on rice in this regard; therefore the development of methods or techniques for isolation of gametes at cellular level and their fusion will be extremely useful. This technological development may be helpful not only in rice but also in other crops. The scope of these procedures has been reviewed by different researchers (Rougier *et al.*, 1996; Kranz and Dresselhaus, 1996; Dumas *et al.*, 1998; Kranz and Kumlehn, 1999).

2.4.1 Isolation of female gametes

The female gametophyte or embryo sac, contains the gametes, and develops within a specialised structure, the ovule. The embryo sac is surrounded by a cellulosic wall, sometimes completed with callous, as in maize (Matthys-Rochon *et al.*, 1992) and this appears to be a major plant structure, a functional unit, in where the original double fertilization occurs (Dumas *et al.*, 1998). To protect the female cells from environmental variations, the embryo sac and gametes are deeply immersed in the diploid female organ (Wagner *et al.*, 1990). Many efforts have been made to isolate embryo sacs and female gamete from mature plants (Theunis *et al.*, 1991). The usual procedure for isolation of female gametes is the enzymatic treatment of ovules, followed by manual manipulation of the embryo sac. Using this technique, several reports have been published for isolation of viable egg cells in several species (Kranz *et al.*, 1991a&b; van der Mass *et al.*, 1993; Kranz and Kumlehn, 1999).

2.4.2 Isolation of male gametes

At maturity, the pollen grain contains either a single progenitor cell or the two sperm cells along with the vegetative cell. This is the classical distinction between the two-celled pollen species (Rosaceae, Solanaceae, etc) and the three-celled pollen species (Cruciferae, Graminaea, etc.). Three-dimensional reconstruction of pollen from several species have shown that in three-celled pollen species, the two sperm cells and the vegetative nucleus were physically associated. The association between the sperm cells' plasma membrane and the nuclear envelope of the vegetative cell was termed the 'male germ unit' (MGU) because of its putative importance in relation to the phenomenon of double fertilisation (Dumas and Mogensen, 1993). This implies that all heredity materials, both cytoplasmic and nuclear, are held together as a single unit (Tian *et al.*, 1998). The validity of this concept was partly demonstrated by isolating this physical unit or MGU (Matthys-Rochon *et al.*, 1987), and also by demonstrating that such an association also exists in the two-celled pollen grain. The main function of the MGU is the transport of male gametes. Their association with the vegetative nucleus appears to facilitate the precise positioning of the two sperm cells in relation to their female target cells (Tian *et al.*, 1998).

Several attempts were made with different species during the period 1985 – 1987 to isolate the MGU. The first significant results were obtained by Russell (1986) and Dupuis *et al.* (1987), who succeeded in isolating sperm cells of *Plumbago* and maize respectively (Dumas *et al.*, 1998). They also verified the viability and integrity of the isolated sperm cells. In the past few years, several cytological procedures have been developed to examine pollen sperm cells in several species (Theunis and Went, 1989; Theunis, 1992; Southworth, 1992; Chaboud and Perez, 1992). These include the use of both light and electron microscopic analyses from sperm cells *in situ* and *in vitro* (Theunis *et al.*, 1991; Southworth, 1992).

All sperm cells have some common features: they are small in size and, after isolation, they become spherical, like vegetative protoplasts. They also contain a very large nucleus and therefore a small volume of cytoplasm. Finally, the isolated sperm cells have no cell wall. The quality of sperm cells has been examined using numerous techniques, including the FCR test (Dupuius *et al.*, 1987), Evans blue staining, ATP measurement (Roeckel-Drevet *et al.*, 1995), or cell sorting (Zhang *et al.*, 1992). There is no doubt that the most absolute assay would be the ability of sperm cell to fuse with a female gamete and to form a zygote.

2.4.3 *In vitro* gametic fusion

In double fertilization, fusion of one sperm cell takes place with the egg cell and the other sperm cell with the central cell (Lopes and Larkins, 1993), in order to develop an embryo and endosperm, respectively. This phenomenon was first reported independently by the Russian Sergius Nawaschin in 1898 and the Frenchman Leon Guignard in 1899 (Dumas *et al.,* 1998). Since then, considerable development has been made in this domain. Both a cellular and a molecular basis of fertilization have been reported, which includes pollen and ovule quality, isolation of sperm and egg cells and their characterisation, and finally their subsequent use for *in vitro* fertilization. Two main strategies for *in vitro* fertilization have been developed in angiosperms during the past few years. One strategy consists of injecting isolated male gametes or male nuclei into isolated embryo sacs (Mathys-Rochon *et al.*, 1994) and the other is induced fusion (either electrofusion or chemically induced fusion). However, the rate of success in injection is very low. In addition, the microinjection of sperm cells into egg cells or central cells completely isolated from surrounding cells has not yet been achieved because of the technical difficulty of the procedure. However, such methods of intra-cytoplasmic sperm injection are already practised in humans (Alikani *et al.*, 1995; Dumas *et al.*, 1998).

The second strategy consists of fusion of gametes, completely isolated from the surrounding tissues, under *in vitro* conditions (Kranz *et al.*, 1991a&b; Kranz and Lorz, 1993, 1994; Faure *et al.*, 1994; Kranz and Kumlehn, 1999). This method consists of aligning pairs of gametes by di-electrophoresis and then fusing them by the use of one or more electric pulses. This method was used successfully for maize (Kranz *et al.*, 1991; Faure *et al.*, 1993; Kranz and Lorz, 1993) and wheat (Kovacs *et al.*, 1995). An alternative to electro-fusion is chemically induced fusion, especially calcium induced fusion, which involves bringing male gametes and egg or central cells into contact with the help of micro-needles (Faure *et al.*, 1994; Kranz and Lorz, 1994). The gametes adhere for a few minutes and then fuse. Unlike electro-fusion, this second *in vitro* system retains gamete specificity (Faure *et al.*, 1994).

Physiological conditions during *in vivo* gamete fusion are unknown. Limited report is available on the osmolarity of the *in vivo* environment surrounding the gametes, its composition, its pH, as well as the temperature at which fusion occurs, although some *in vitro* reports are available. A comparison of the physiological state of gametes *in vitro* and *in vivo* is important for fertilization experiments. Gametes must be put into close contact to allow *in vitro* fusion. Under *in vivo* conditions, cytoskeletal elements in the embryo sac might be responsible for the apposition of the male gametes to the egg and central cells. It is reported that synergids might play a significant role in this regard, as they enrich with calcium.

Observation suggests that calcium concentration might be essential for gamete survival and fusion, as Faure *et al.* (1994) reported that the highest fusion is obtained at a higher concentration of calcium in maize. Therefore it seems that calcium in the millimolar range is essential for gametic fusion, which is also observed in other organisms such as mammals (Dumas *et al.*, 1998). The concentration of bound calcium is high in the synergids of grass species (Chaubal and Reger, 1990) and, after synergid degeneration around the egg cell, may lead to gametic fusion (Huang and Russell, 1992; Chaubal and Reger, 1993). It is also reported that calcium concentration is important for egg cell membrane stability in

barley (Holm *et al.*, 1994) and for sperm cell viability in maize (Zhang *et al.*, 1995).

For *in vitro* fusion, it is a prerequisite to bring gametes into close contact. This can be done either by dielectrophoresis (Kranz *et al.*, 1991a & b) or using microneedles and allowing the gametes to adhere to each other. Under *in vivo* conditions, embryo sac elements might be responsible for the apposition of the male gametes to the egg and central cells. The involvement of these structures in pulling the male gametes towards their targets still needs to be investigated and demonstrated. The molecules involved in the later adhesion of the gametes are unknown. Several studies have led to the identification of such molecules in animal species (Rougier *et al.*, 1996) as well as in algae, e.g. *Fucus* (Wright *et al.*, 1995). This phenomenon in angiosperm gametes may be widespread. Indeed, male gametes can also adhere to each other and to mesophyll protoplasts, although their fusion is not frequent (Faure *et al.*, 1994). *In vitro* fertilization without electrical pulses could be used as a bioassay to identify molecules involved in the adhesion of gametes.

Gametic fusion occurs rapidly. Male gametes fuse with their counter-part very rapidly (Faure *et al.*, 1994; Kranz and Lorz, 1994). Indeed, the fusion of the gametes has rarely been observed by electron microscopy, indicating that this is a rapid event (Russell, 1992). This suggests the absence of preferential fertilization. In other words, the male gametes are equally as likely to fuse with the egg cell or the central cell. In this respect, *Plumbago zeylanica* is different (Russell, 1993). In this species, the two male gametes from one pollen grain are dimorphic: one is plastid-rich and fuses more frequently with the central cell (Russell, 1993). Another aspect of gamete recognition is whether a barrier to interspecific crosses exists at the gamete plasma membrane level. So far, the electrofusion of maize egg cells with sorghum, *Triticum Hordeum* and *Brassica* male gametes have been reported without any complication (Kranz *et al.*, 1995; Kranz and Dresselhaus, 1996; Kranz and Kumlehn, 1999). However, the question of interspecific crossability is not addressed, as electric pulses forced the fusion in the above

examples. Corresponding *in vitro* fertilization without electro-fusion is therefore required. The potential implications of this *in vitro* fertilization are important. Because the fusion parameters can be controlled and the timing is precise, the study of gamete adhesion, fusion and signalling should now be feasible. In addition, *in vitro* protocols can be combined with new molecular tools.

2.4.4 Plant regeneration from fused gametes

The application of micromanipulation techniques enables isolation and *in vitro* fusion of female and male gametes in angiosperm. In combination with tissue culture methods, which are adapted for the culture of single cells, these techniques allow individual development of zygotes and endosperm. Zygotes and endosperm are able to self-organise in culture independently from maternal tissue. Kranz and Kumlehn (1999) have reported regeneration of plants from the zygotes produced *in vitro* fertilization and from *in vivo* pollination. Kranz and Lorz (1993) have reported regeneration of fertile maize plants from fused gametes using 0.1 or 0.5 mg/l 2, 4-D, 1.0 mg/l NAA, 1.0 mg/l benzyladenine during the first phase, and, for later stages, an MS medium without hormones and 40-60 mg/l sucrose in MS medium. Ability of zygotes to organise and develop into plants may be used for the transfer of useful genes from one species to other.

2.5 Rationale and significance of the study

The world's population is expected to increase by about three billion by the first quarter of the new millennium. This has a special impact on developing countries as population growth rates are still higher compared to developed countries, as mentioned in chapter one. This implies that rice production must be increased by about 50% (say about 300 million tons) from the present level to feed the projected population. Cultivation of improved rice varieties with high and stable yield potential is the best policy to keep pace with ever-growing populations. Rice is grown on about 148 million hectares of lands in the world and about 40 and 20 million hectares are under rainfed and upland conditions respectively, where drought at some stages of

the life cycle limits yields, sometimes seriously. Moreover, productivity is low (4-5 t/ha) in rainfed and upland ecosystems compared to irrigated (5-6 t/ha) ecosystem (BRRI, 1997). It was apparent that rice production could be increased in these two ecosystems if appropriate varieties can be developed. Therefore a project has been undertaken to identify drought resistant type(s) with the aim to utilise this in rice breeding programmes through the development of novel hybridization methods.

In order to do this, the identification of drought resistance type(s) was carried out using physio-morphological parameters and isozyme systems. Therefore pot plant screening experiments were carried out to identify drought resistant accessions, under greenhouse conditions according to physio-morphological characteristics and through isozyme study in the laboratory. Attempts were then made to develop methods for isolation of gametes and for their fusion. Plant regeneration from fused gametes was also carried out with the aim to facilitate gene transfer.

CHAPTER THREE

Selection of drought resistant accessions

3.1 Introduction

Rice (*Oryza sativa* L.) is a semi-aquatic plant and grows in a wide range of environments, from rainfed to wetland conditions. In rainfed area, the soil is largely aerobic, and plants depend on rainfall, whereas in wetland, the soil is puddled, flooded, anaerobic and most of the season remains under water (O'Toole and Chang, 1979; Turner *et al.*, 1986).

Crop plants rarely show their total genetic potential for yield, due to limitations imposed by environmental factors. Unfavourable temperature and water deficit are the most important limitations affecting crop yields. About one-third of the world's potential cultivable land suffers from an inadequate supply of water and/or in drought conditions (David, 1991; Price *et al.*, 1997). Moreover, most of the remaining areas' crop yields are periodically reduced by drought. The severity and time of such stress can differ from season to season and year to year. Its effect is often amalgamated with other abiotic and/or biotic stresses (Clover *et al.*, 2001).

Hydrological conditions interact with edaphic, biotic, agronomic and other climatic factors, and agricultural crop productivity is dependent on the complex interactions of plants with those factors. These interactive phenomenon influence the adaptation of rice cultivars in different ecology. This has special implications for rainfed and dry land areas. It is estimated that about half of the world's rice is cultivated in rainfed and upland areas where several unfavourable features including inadequate water limits crop production (Bennett, 2001; Pantuwan *et al.*, 2002). The range of water stress environments is wide and can affect the crop at any stage of growth. Therefore a rice variety, which is resistant to drought, is a useful asset to increase productivity.

Drought resistance has been attributed to larger root systems, an ability to delay reproductive development and an ability to maintain stomata opening at low levels of leaf water potential, possibly through osmotic adjustment (Loresto *et al.*, 1976; Wright *et al.*, 1983; Hsiao *et al.*, 1984; Azam-Ali and Squire, 2002). The delay in reproduction is not always beneficial to the farmer. In reality, as mentioned earlier, drought resistance is the result of the interaction of numerous morphological, anatomical and physiological characteristics and different authors have explained the above-mentioned phenomenon in different ways (Wright *et al.*, 1983, Turner *et al.*, 2001). This makes difficult for researchers to understand the importance of stress response, when attempting to develop breeding strategy for stress resistant varieties.

Generally in cereals, field resistance to drought is associated with low tillering ability, tall plant stature, deep and thick roots, and plasticity of leaf rolling and unrolling. On the other hand, recovery from drought is associated with good tillering ability, and low plasticity of leaf rolling and unrolling (Chang *et al.*, 1982). Therefore it is important from the breeding point of view to consider the above characteristics at the time of selection or when designing a breeding programme. The development of drought resistant varieties has been slow although information is emerging on stress physiology. However, compared to other major cereals limited information is available on the response of rice to water deficits.

One of the major causes for slow progress in developing drought resistant varieties of rice is the influence of the environment, which arises from a combination of factors such as genotype and environmental (GxE) interactions. Plant breeders have tried to interpret how the environmental conditions influence genotypes or how genotypes respond to varying environments. Unfortunately, the influence of the environment over genotypes and the model for yield provides little understanding of the biological significance (Turner *et al.*, 2001). This complicates the selection of new varieties or cultivars in drought conditions, as drought resistance itself is an ambiguous term, as described in Chapter Two. The physiological basis of GxE interaction could therefore help to understand the adaptations of suitable genotypes for yield and quality. It is apparent that

development of drought resistant varieties at different stress conditions may require different breeding approaches. Therefore, a pot plant-screening experiment with the germplasm obtained from Bangladesh was carried out to identify drought resistant accessions, which could be used in rice improvement programme.

3.2 Materials and Methods

Pot plant screening experiments were carried out in a glasshouse of the University of Southampton during the period of July-September, 1998 and May-August, 1999. The glasshouse climatic data recorded and collected from meteorological centre during the experimental periods are shown in Fig. 3.1a and Fig.3.1b. A total of 21 and 51 accessions of rice were used in experiment-1 and experiment-2, respectively. The accessions were considered as treatments in both experiments. The experiments were carried out in a Randomised Complete Block Design (RCBD) with 4 replications. Rice seeds were sown in plastic pots of 15-cm diameter with a surface area of 177 cm^2. Pots were filled with 885 cm^3 of soil (John Innes compost number 2), which contained plant nutrients of NPK and other macro and microelements. The rice accessions that were used in the experiments were obtained from the Bangladesh Rice Research Institute (BRRI) Genebank (Table 3.1). The germplasm was collected from different ecotypes and rainfall distribution pattern of Bangladesh, which is given in Table 3.1. Eight seeds of each accession were dibbled in 2-cm depth in each pot. Shallow irrigation (200cm^3/pot) was applied after sowing. After the emergence of seedlings from the soil the pots were irrigated twice a week with water (200 cm^3/pot) until seedling establishment (*i.e.* seedlings had sufficiently developed roots to extract nutrients from the soil and 2-3 leaves had emerged to begin the important function of photosynthesis).

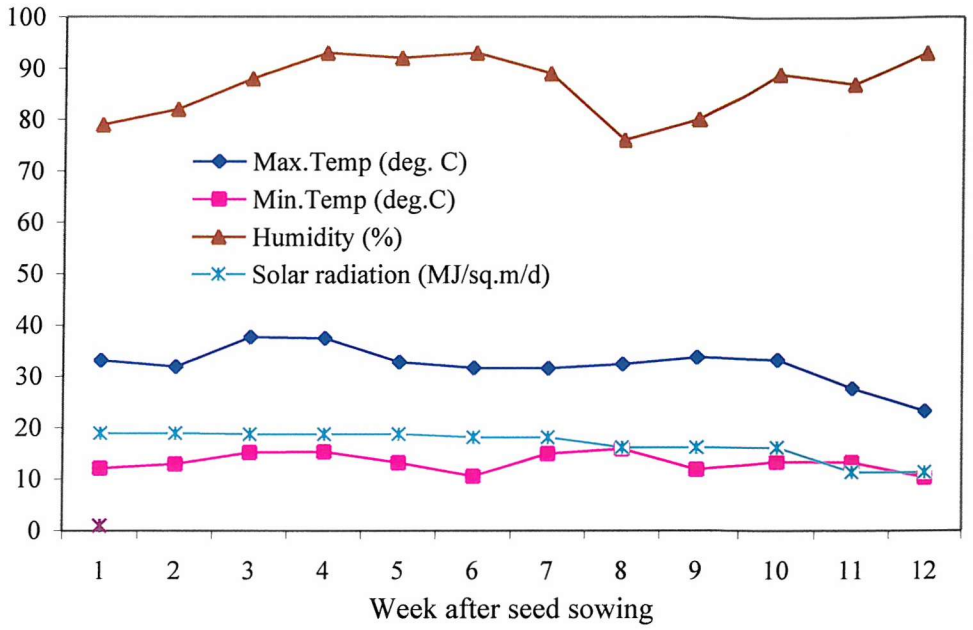

Fig. 3.1a Available climatic data for experiment-1 (1998)

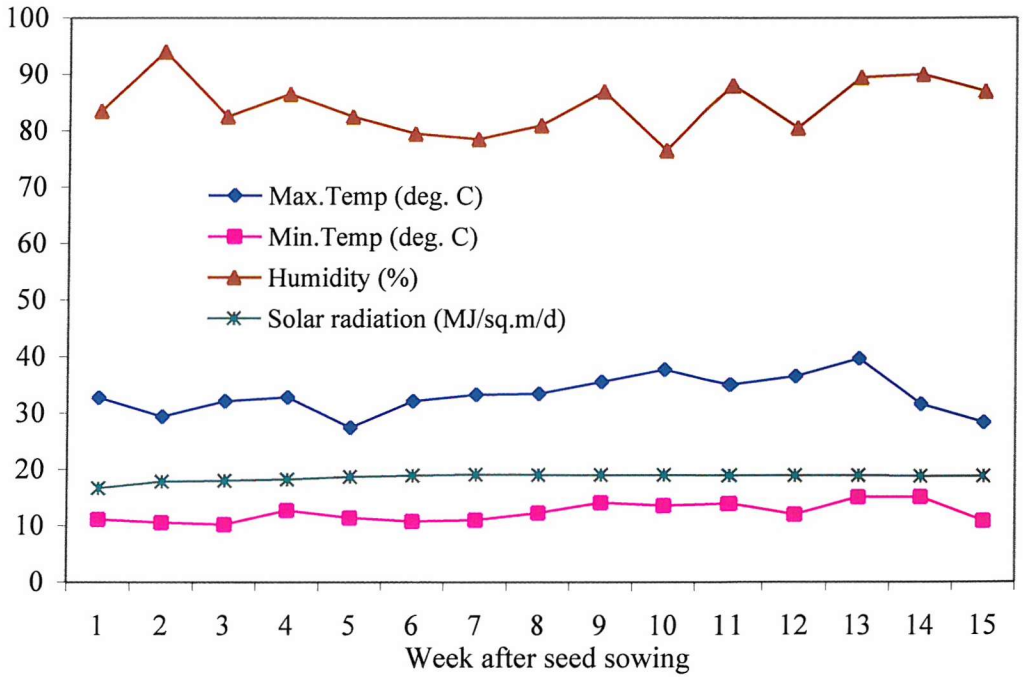

Fig.3.1b Available climatic data in experiment-2 (1999)

Table 3.1 List of rice accessions with accession number and provenance, which were used in the experiments

Serial number	Name of accession	BRRI accession number	Growing season*	Collection district	Collection region***	Rainfall pattern[a]
1	Dharial	0018	Aus/Upland	DA-14**	-	Moderate rainfall
2	Dular	0022	Aus/Upland	DA-22	-	Moderate rainfall
3	Hashi Kalmi	0030	Aus/Upland	DA-23	-	Moderate rainfall
4	Kataktara	0039	Aus/Upland	DA-2	-	Moderate rainfall
5	Marichbati	0047	Aus/Upland	DA	-	Moderate rainfall
6	Panbira-1	0050	Aus/Upland	DA-12	-	Moderate rainfall
7	Hijolee	0571	Aus/Upland	Rangpur	Rangpur	Low/moderate rainfall
8	Aus Baku	1318	Aus/Upland	Kustia	Meherpur	Low/moderate rainfall
9	Hasha	1534	Aus/Upland	Dinajpur	JhikorGacha	Low/moderate rainfall
10	Manik Mondal	1692	Aus/Upland	Faridpur	-	Moderate rainfall
11	Huma Gambir	1738	Aus/Upland	Khulna	-	Moderate rainfall
12	Hanumanjata	1739	Aus/Upland	Khulna	-	Moderate rainfall
13	Boalia	2068	Aus/Upland	Kishoregonj	Hossainpur	Moderate/heavy rainfall
14	Ausa Bogi	2075	Aus/Upland	Kishoregonj	Kendua	Moderate/heavy rainfall
15	Agali	2082	Aus/Upland	Netrokona	Netrokona	Heavy rainfall
16	Bogi	2083	Aus/Upland	Netrokona	Netrokona	Heavy rainfall
17	Kumari Aus	2100	Aus/Upland	Jamalpur	Dewangonj	Heavy rainfall
18	Gopal Bhog	2109	Aus/Upland	Narsingdi	Narsingdi	Moderate rainfall
19	Sada Aus	1235	Aus/Upland	Pabna	Pabna	Low rainfall
20	Hazi Faram	2150	Aus/Upland	Rajshahi	Charghat	Low rainfall
21	Bina Muri-1	2181	Aus/Upland	Bogra	-	Moderate rainfall
22	Bina Muri-2	2184	Aus/Upland	Bogra	-	Moderate rainfall
23	Bakee	2358	Aus/Upland	Jamalpur	-	Moderate/heavy rainfall
24	Boila Bokri	3194	Aus/Upland	Munshigonj	Lauhajong	Moderate rainfall
25	Aus Nagra	3455	Aus/Upland	Jessore	Navaron	Low/Moderate rainfall
26	Aug Meghi	3456	Aus/Upland	Jessore	Navaron	Low/moderate rainfall
27	Bok Tulsi	3461	Aus/Upland	Shatkhira	Sadar	Moderate rainfall
28	Aus Kushi	3501	Aus/Upland	Shatkhira	Tala	Moderate rainfall
29	Bali Guri	3502	Aus/Upland	Mymensingh	Haluaghat	Heavy rainfall
30	Binna Toa	4197	Aus/Upland	Noakhali	Sonagazi	Heavy rainfall
31	Hogla Pata	3871	T. Aman	Barisal	-	Moderate rainfall
32	Kada Moni	0573	Aus/Upland	Rangpur	-	Low/moderate rainfall
33	Kala Mona	0984	T. Aman	Comilla	Baliaghata	Moderate rainfall
34	Kumra Gair	3878	T. Aman	Barisal	-	Moderate rainfall
35	Kacha Mota	3879	T. Aman	Barisal	-	Moderate rainfall
36	Kartik Sail	3662	T. Aman	Sherpur	Sherpur	Heavy rainfall
37	Kola Mocha	4141	B. Aman	Jhenidah	Jhenidah	Low/moderate rainfall
38	Lakhai	1800	Boro	Kishoregonj	Tarail	Moderate/heavy rainfall
39	Nuncha	0942	Aus/Upland	Khulna	Fakirhat	Moderate rainfall
40	Nona Balam	3203	T. Aman	Barisal	-	Moderate rainfall
41	Panbira-2	4150	T. Aman	Khulna	Fultola	Moderate rainfall
42	Tilock Kachari	0758	T. Aman	Chittagong	Boalkhali	Heavy rainfall
43	Aswina	0927	T. Aman	Sylhet	-	Heavy Rainfall
44	Dud Kalam	0278	T. Aman	Rangpur	Sundargonj	Low/moderate rainfall
45	Keora	0731	B. Aman	Comilla	-	Moderate rainfall
46	Hogla	4178	T. Aman	Jessore	-	Moderate rainfall
47	Kumari	0203	T. Aman	-	-	-
48	Dhapa	0320	T. Aman	Rangpur	Hatibandha	Low rainfall
49	Raja Sail	0758	T. Aman	Chittagong	Sitakunda	Heavy rainfall
50	Dud Mona	3862	T. Aman	Barisal		Moderate rainfall
51	Kajal Sail	0612	T. Aman	Noakhali	Sonagazi	Heavy rainfall

[a]Low Rainfall <1600mm, Moderate Rainfall 1600-2500 mm, Heavy Rainfall >2500mm

- Data not available, * Rice growing ecosystem, Aus = Summer rice, T.Aman = autumn/rainfed lowland rice, Boro = winter/irrigated rice (Oka, 1991) **DA means Dhaka Agricultural station collection number ***Administrative unit

After the establishment of seedlings, they were thinned out to only 5 seedlings per pot, and irrigation was reduced, applied once a week until artificial water stress was imposed. At the vegetative stage (before panicle primordial initiation), water stress was imposed after 45 days of emergence and visual score was made for the degree of drought intensity. The plants were scored for drought resistance after 15 days of imposed stress according to the method followed by O'Toole and Maguling (1981) and the Standard Evaluation System for rice of the International Rice Research Institute (IRRI, 1996a). The scale was from 0 to 9, where 0 is higher drought resistance (healthy leaves, no visible leaf rolling or necrosis) and 9 is the highest drought susceptibility (plants apparently dead). After measuring the visual drought score, pots were watered again once a week. The stomata conductance (cms^{-1}) on abaxial leaf surface was measured using a diffusive resistance automatic porometer (Delta-T device, Mark II, Cambridge, England) just prior to the stress period (37 days after emergence) and at the stress period (52 days after emergence). Measurement was made of the youngest fully expanded leaf of five plants in each treatment. The average stomata density (per sq. mm) was observed (5 observations per plant) under a stereomicroscope. Plant heights were measured from the ground base to the end of the tallest leaf. Leaf length from leaf collar to the tip of the leaf and leaf width was measured from randomly selected plants at the maximum tillering stage of plant growth. Leaf area (cm^2) was calculated by using Leica-Q-win image processing and analysis system (Lecia-imaging system ltd., Cambridge, England).

Pots for the measurement of root length and dry weight of root were selected and were washed with water to remove the soil adhering to roots. The weight of oven dried (constant weight at 80°C) shoot and root per plant was measured. The ratios of root-to-shoot length (root length/shoot length) and their dry weight (root dry weight/shoot dry weight) were calculated. Tiller numbers per plant; from randomly selected plants were counted. The number of days required to seedlings emergence was recorded.

Total seasonal and daily average evapotranspiration (ET) was estimated over intervals of one week by using the following water balance equation:

$$ET = \{I + (S_1 - S_2)\}/\text{surface area of pot}$$

Where I= irrigation, $(S_1 - S_2)$= the initial and final weight of pot (soil water contents) for the calculated period.

Every pot was weighed once a week (of the same day of the week) in order to determine water loss by evapotranspiration. However, in experiment-1, data were obtained for only four weeks.

Water use efficiency (WUE) was measured in terms of the ratio of dry matter produced or crop yield to water used in transpiration and soil evaporation (Kramer, 1980). The following formula was used for the calculation of WUE.

WUE = dry matter or crop yield/evapotranspiration (g/kg)

3.2.1 Data analysis

Analysis of variance and covariance, coefficient of variation (CV), simple linear regression and Pearson's bivariate correlation were used in order to measure the inter-relationships among the drought-related parameters. Duncan multiple range test (DMRT) was carried out to compare the treatment means. Alphabetic letters were used for ranking of the differences between treatments. Any two means having a common letter were not significantly different at the 5% level and a different letter indicated means were significantly different.

To identify drought resistant accessions, a cluster analysis was conducted with an agglomerative hierarchical clustering method (HCA), and a rescaled distance similarity measure was used for cluster analysis. Cluster analysis is one of the wide ranges of general procedures used to create a classification and each cluster or group is formed with highly similar entities. Initially, a factor analysis was carried out of the data matrix.

For the factor analysis, the Principal Component Analysis (PCA) was chosen, as this is a simple method and widely used, which can be employed to reduce and purify the variables with a conceptually more coherent set of variables. In general, the first step of the analysis involved an examination of the interrelationships among the variables. The principal component scores with eigenvalues, which were greater than, or equivalent to 1.0, were used as new variables for cluster analysis. In Hierarchical Cluster Analysis (HCA), Ward's linkage was used for clustering and the Squared Euclidean distance method was used for dissimilarity measures. The original data was transformed to Z-scores prior to cluster analysis. All analyses were carried out using the Excel and the SPSS software programme version 10.

3.3 Results

3.3.1 Experiment-1

Twenty-one rice accessions were evaluated in the glasshouse for the observation of drought resistance based on their physio-morphological properties in experiment-1. Table 3.2 shows the grand means, maximum and minimum values of treatment means, one sigma interval estimation of means, coefficient of variation (CV) and calculated F values (Fischer significant test value), based on two-way analysis of variance of all parameters. The tabulated F values with 20, 60 degrees of freedom (*d.f.*) are 1.75 at 5% level and 2.20 at 1% level of significance. The calculated F values for 20, 60 *d.f.* (F values from 1.81 to 19.49) are greater than the tabulated F values in all parameters. Hence the treatment means are significantly different even at the 1% level of significance in all parameters recorded in this experiment indicating a wide range of variability where drought resistant accessions might be identifiable. Large differences observed in the parameters of the descriptive statistics, such as maximum and minimum values indicated the existence of diversity in the germplasm.

Visual drought scores made after 15 days of cessation of irrigation indicated that susceptible accessions could be identified visually compared to the resistant ones for the degree of leaf rolling, since they had tightly rolled or drying leaves. The observations indicated large differences between the treatment means, as it appeared from Duncan Multiple Range Test (DMRT) that most of the accessions showed different ranking at 5% level (Table 3.3 and Appendix: Table 3.1). This indicated that some accessions could be a potential source for drought resistance. The largest tabulated shortest significant range of DMRT for 60 *d.f.* is 0.54 and the difference between the largest treatment mean and the highest shortest significant range is 7.34, which is greater than any other treatment means. Hence, the equality assumption of accessions (null hypothesis) was rejected at 5% level and it was concluded that there were significant differences between the accessions. Similar interpretation can be made in other treatment means. The accession Kumra Gair had the highest mean value of visual drought score (mean 7.88 and SE= 0.47) and

the accession Keora had the lowest mean value (mean 1.56 and SE= 0.21) when visually scored for drought. At least five accessions showed lower drought score values, which were statistically similar and could possess drought resistant characteristics, since the leaves of these accessions were completely unrolled at morning, rolled at midday, and again unrolled in late afternoon.

Table 3.2 Descriptive statistics and F values of different physio-morphological characters based on two-way analysis of variance in experiment-1

Characters	Minimum	Maximum	Mean ±SE	CV (%)	F-Value
Drought score	1.00	8.50	3.83±0.22	11.49	5.76**
Days to emergence	6.00	19.00	11.00±0.32	10.79	19.49**
Tiller number per plant	2.60	9.00	4.94±0.15	19.14	4.48**
Plant height (cm)	42.75	73.60	56.55±0.65	8.01	4.07**
Root length (cm)	14.75	45.90	29.86±0.91	20.27	3.58**
Root-shoot length ratio	0.26	0.80	0.53±0.11	28.65	8.17**
Stomata conductance (cm/s) prior to stress	0.16	0.55	0.30±0.01	28.11	5.08**
Stomata conductance (cm/s) at stress	0.04	0.11	0.062±0.01	32.70	2.22**
Stomata number	85	190	135±2.50	16.97	1.90*
Leaf length (cm)	29.90	46.94	38.62±0.38	8.99	4.67**
Leaf width (cm)	0.65	1.05	0.80±0.01	12.01	11.74**
Leaf area (cm^2)	11.39	25.66	18.20±0.36	16.44	6.04**
Root dry weight (g)	0.13	0.67	0.37±0.02	21.80	2.73**
Shoot dry weight (g)	0.74	3.42	1.57±0.06	26.59	2.20**
Total dry mass (g)	1.02	3.74	1.94±0.07	26.69	2.26**
Root-shoot dry weight ratio	0.09	0.54	0.25±0.10	42.95	4.30**
ET (mm/day)	2.31	4.37	3.41±0.09	13.24	1.81*
WUE (g/kg)	1.21	2.49	1.85±0.02	30.11	2.01*

SE= Standard error of mean ** Significant at 1% level, *Significant at 5% level, F value= Fischer significant test value

Correlation coefficient between drought score and root length ($r=-0.44$, $p<0.001$, $N=84$), dry weight of root ($r=-0.23$, $p=0.037$, $N=84$), and root-shoot length ratio ($r=-0.40$, $p<0.001$, $N=84$) were significant (Table 3.4). T-test also showed significant correlation at 5% level betweens drought score and root length, drought score and dry weight of root, drought score and root-shoot dry weight ratio, drought score and root-shoot length ratio. This implied that drought score was mostly related to root length, root-shoot length ratio, and dry weight of root.

Table 3.3 Summarised mean results of drought related parameters and one standard deviation of promising drought resistance accessions of rice in experiment-1

Name of Accessions	Drought score	Stomata number (per sq. mm)	Conductance (cm/s) prior to stress	Conductance (cm/s) at stress	Leaf length (cm)	Leaf width (cm)	Leaf area (per cm^2)	Shoot dry weight (g) per plant	Root dry weight (g) per plant	Root-to-shoot dry weight ratio
Keora	1.56±0.43a	134abc	0.38±0.05e	0.05±0.006b	39.95±1.75d-g	0.93±0.04g	21.74±1.89g	1.25±0.29a	0.51±0.06de	0.42±0.10de
Dhapa	1.63±0.63a	147fghabc	0.34±0.08c	0.04±0.01a	37.15±4.05a-e	0.90±0.09g	19.66±3.88c	1.86±0.69ab	0.53±0.09e	0.31±0.05bcd
Tilock Kachari	1.69±0.75a	149abc	0.40±0.13e	0.06±0.01b	38.47±2.07a-e	0.78±0.03bcde	17.65±0.74bc	1.54±0.39ab	0.46±0.05de	0.31±0.05bcd
Kala Mona	1.75±0.29a	141abc	0.35±0.02de	0.06±0.01b	40.61±2.30efg	0.86±0.04efg	20.38±1.77e	1.61±0.32ab	0.54±0.06e	0.35±0.08cde
Dud Kalam	1.75±0.29	139abc	0.38±0.03e	0.04±0.01b	40.44±2.41efg	0.76±0.05bcd	18.07±1.22bc	2.24±0.50b	0.47±0.11de	0.19±0.07ab
Kada Moni	3.00±1.15cd	135abc	0.37±0.07e	0.04±0.005b	35.79±1.68a-d	1.02±0.02h	21.39±1.16f	1.24±0.13a	0.54±0.07e	0.44±0.04e
Dud Mona	3.25±2.06a-d	124abc	0.27±0.05b	0.04±0.01b	39.93±1.35d-g	0.81±0.01def	19.04±0.74bc	2.12±0.61b	0.37±0.05d	0.19±0.05ab
Hogla Pata	3.75±1.32a-d	159c	0.37±0.09e	0.07±0.03c	34.45±2.09a	0.67±0.02a	13.62±1.08a	1.65±0.22ab	0.37±0.08d	0.23±0.03abc
Panbira-2	4.63±2.52bcd	131abc	0.26±0.06b	0.07±0.02c	39.41±2.49c-f	0.74±0.04abcd	17.12±1.87bc	1.59±0.12ab	0.25±0.12ab	0.15±0.07a
Kumra Gair	7.88±0.95e	121ab	0.21±0.02a	0.08±0.01c	40.87±2.20efg	0.85±0.03efg	20.35±1.72e	1.69±0.44ab	0.32±0.17bc	0.20±0.14ab
SE	0.22	3.00	0.01	0.01	0.38	0.01	0.36	0.06	0.02	0.10
DMRT*	0.54/0.44	7.08/5.77	0.03/0.02	0.005/0.004	0.91/0.76	0.03/0.02	0.88/0.72	0.15/0.12	0.05/0.04	0.03/0.02

SE= Standard error of mean, Mean followed by alphabet showing DMRT ranking: Any two means having a common letter are not significantly different at the 5% level

* Largest/smallest shortage significant range values

Table 3.4 Pearson correlation coefficient among the drought related parameters in experiment-1

	Visual Drought Score	Stomata conductance prior to stress	Stomata conductance at stress	Stomata number per sq. mm	Leaf length (cm)	Leaf width (cm)	Leaf area per sq. cm	Shoot length (cm)	Root length (cm)	Root to shoot length ratio	Shoot dry weight (g)	Root dry weight (g)	Root-shoot dry weight ratio	Total drymass (g)	Evapotranspiration (mm/day)	Water use efficiency (g/kg)	Tiller /plant	Days to emergence
Drought Score	1	-0.30**	0.16^{NS}	-0.12^{NS}	0.05^{NS}	-0.17^{NS}	0.01^{NS}	-0.06^{NS}	-0.44**	-0.40**	-0.08^{NS}	-0.23*	-0.21*	-0.14^{NS}	-0.15^{NS}	-0.03^{NS}	-0.17^{NS}	-0.47**
		.006	.134	.281	.666	.132	.901	.599	.000	.000	.458	.037	.048	.201	.189	.782	.124	.000
St. conductance prior to stress		1	0.008	0.62**	-0.05^{NS}	0.14^{NS}	0.33**	0.08^{NS}	0.31**	0.26*	0.21^{NS}	0.33**	0.27*	0.31**	0.15^{NS}	0.33**	0.11^{NS}	0.21*
			.945	.000	.646	.222	.002	.477	.004	.015	.052	.003	.013	.004	.181	.002	.311	.050
St. cond. at stress			1	0.01^{NS}	0.07^{NS}	-0.24*	0.09^{NS}	0.06^{NS}	-0.32**	-0.32**	-0.09^{NS}	-0.27*	-0.19^{NS}	-0.14^{NS}	-0.10^{NS}	-0.11^{NS}	-0.07^{NS}	-0.18^{NS}
				.923	.502	.029	.76	.595	.003	.003	.434	.012	.069	.208	.371	.322	.525	.102
St. number				1	-0.09^{NS}	-0.20^{NS}	0.38**	0.004^{NS}	0.06^{NS}	0.04^{NS}	0.25	0.08^{NS}	-0.06^{NS}	0.24*	0.20^{NS}	0.13^{NS}	0.18^{NS}	0.17^{NS}
					.410	.063	.000	.971	.563	.735	.020	.485	.622	.025	.058	.239	.095	.115
Leaf length					1	0.27*	0.38**	0.77**	-0.05^{NS}	-0.31**	0.16^{NS}	-0.003^{NS}	-0.11^{NS}	0.16^{NS}	0.13^{NS}	0.08^{NS}	-0.07^{NS}	-0.14^{NS}
						.012	.000	.000	.649	.004	.156	.977	.301	.133	.249	.454	.546	.208
Leaf width (cm)						1	0.09^{NS}	0.33**	0.36**	0.24*	-0.10^{NS}	0.40**	0.46**	0.02^{NS}	-0.12^{NS}	0.19^{NS}	-0.11^{NS}	0.22*
							.55	.002	.001	.028	.353	.000	.000	.884	.283	.091	.344	.050
Leaf area							1	0.38**	0.04^{NS}	-0.11^{NS}	0.65**	0.24*	-0.16^{NS}	0.67**	0.61**	0.34**	0.23*	0.02^{NS}
								.000	.728	.32	.000	.026	.134	.000	.000	.005	.039	.853
Shoot length (cm)								1	0.05^{NS}	-0.31**	0.23*	0.08^{NS}	-0.05^{NS}	0.25*	0.22*	0.10^{NS}	-0.04^{NS}	0.14^{NS}
									.642	.004	.032	.488	.685	.024	.049	.354	.709	.216
Root length									1	0.93**	0.009^{NS}	0.61**	0.55**	0.16^{NS}	0.09^{NS}	0.19^{NS}	0.002^{NS}	0.37**
										.000	.932	.000	.000	.135	.390	.072	.988	.000
Root-shoot length ratio										1	-0.08^{NS}	0.54**	0.52**	0.06^{NS}	0.01^{NS}	0.14^{NS}	0.001^{NS}	0.30**
											.455	.000	.000	.602	.929	.209	.993	.006
Shoot dry weight (g)											1	0.16^{NS}	-0.37**	0.96**	0.78**	0.56**	0.65**	0.11^{NS}
												.139	.001	.000	.000	.000	.000	.316
Dry weight of root (g)												1	0.78**	0.42**	0.23*	0.45**	0.06^{NS}	0.19^{NS}
													.000	.000	.033	.000	.594	.069
Root-Shoot dry weight ratio													1	-0.20^{NS}	-0.30**	0.09^{NS}	-0.37**	0.10^{NS}
														.056	.005	.425	.001	.361
Total drymass (g)														1	0.78**	0.65**	0.62**	0.12^{NS}
															.000	.000	.000	.261
ET (mm/day)															1	0.05^{NS}	0.52**	0.20^{NS}
																.658	.000	.063
WUE (g/kg)																1	0.32**	-0.04^{NS}
																	.003	.723
Tiller/plant																	1	0.28**
																		.01
Days to emergence																		1

D= Drought, St = Stomata, Cond= conductance, *Significant at P<0.05 and ** Significant at P<0.01, ^{NS} Not Significant at P =0.05 & 0.01, N=84

The correlations between mean drought score and shoot dry weight, shoot length, total dry weight and tiller number were not significant and were negative (Table 3.4). This indicated that larger root length and higher dry weight of roots were more important characters for selection than the dry mass of the shoot for drought resistance. Thus the accessions with favourable root characteristics, such as higher dry mass of root, larger root length and greater root-shoot dry weight ratio might be potential source for drought resistance. Finally, Table 3.3 and Appendix: Table 3.2 revealed that the accessions Tilock Kachari, Kala Mona, Keora, Kada Moni, Dhapa and Dud Kalam had larger root length and dry mass of root, as well as higher ratio of root-to-shoot length and dry mass and these accessions may therefore possess drought resistance characteristics.

The mean values of leaf stomata conductance and stomata number, leaf length, width and leaf areas are shown in Table 3.3 (details in Appendix: Table 3.3). The results showed that there were significant differences in leaf related parameters between the accessions as they appeared from the analysis of variance and DMRT (Tables 3.2 and 3.3). The largest treatment mean is 159 in accession Hogla Pata and the maximum shortest significant range for stomata number is 7.08. The difference between the largest treatment mean and the maximum shortage significant range is 151. This indicated significant difference (5% level) in the treatment means, which resulted from DMRT ranking. Similar interpretation can be made in other leaf related parameters. The accessions, which had higher stomata conductance prior to stress period, had considerably lower conductance in water stress conditions. This suggested that the above-mentioned accessions had the capacity to maintain water balance under stress conditions. In this study, at least seven accessions were found to possess such characteristics, and could be considered for selection for drought resistance.

Covariance analysis using dry mass as the covariate showed significant differences in treatment means of evapotranspiration (ET) (Table 3.2). The points plotted below in the scatter diagram in Figure 3.2 are accessions based on daily average evapotranspiration (ET) and water use efficiency. It appears that the accessions have a slight tendency for higher water use efficiency with higher evapotranspiration. The accessions, which had higher water use efficiency for a

given ET value, could be considered to have useful traits for selecting drought resistant accessions/varieties. Fig. 3.2 shows that about 38% of the accessions fell below the 1:1 line, which implied that 38% of accessions used water more efficiently with lower ET and these accessions could be considered as promising drought resistance types from this study. It was apparent that the accessions Dhapa, Kada Moni, Kala Mona, Dud Kalam, Keora, Hogla Pata, and Dud Mona could possess the above characteristics and could be selected for drought resistance (Appendix: Table 3.1) as these had the capacity for increasing dry matter production through efficient water use.

Similarly, the results obtained from experiment-1 show that the total dry mass production was also related to ET and water use efficiency (Figs. 3.3 and 3.4). Generally, it appears that dry matter production increases in the accessions (slope 1.01x, 0.48x) with ET and water use efficiency (Figs. 3.3 and 3.4) respectively and the regression lines showed that ET ($R^2 = 0.72$) and water use efficiency ($R^2 = 0.71$) is strongly associated with dry mass. Similar interpretation can be made from regression models (Appendix: Tables 3.4 and 3.5). However, Figs. 3.3 and 3.4 show that some accessions had higher dry matter production with lower ET and this is possibly due to less water use. The above phenomena may be considered as one of the mechanisms for drought avoidance since the plants were able to reduce water loss by opening and closing of stomata. These results are in agreement with that of Turner *et al.* (1986&a).

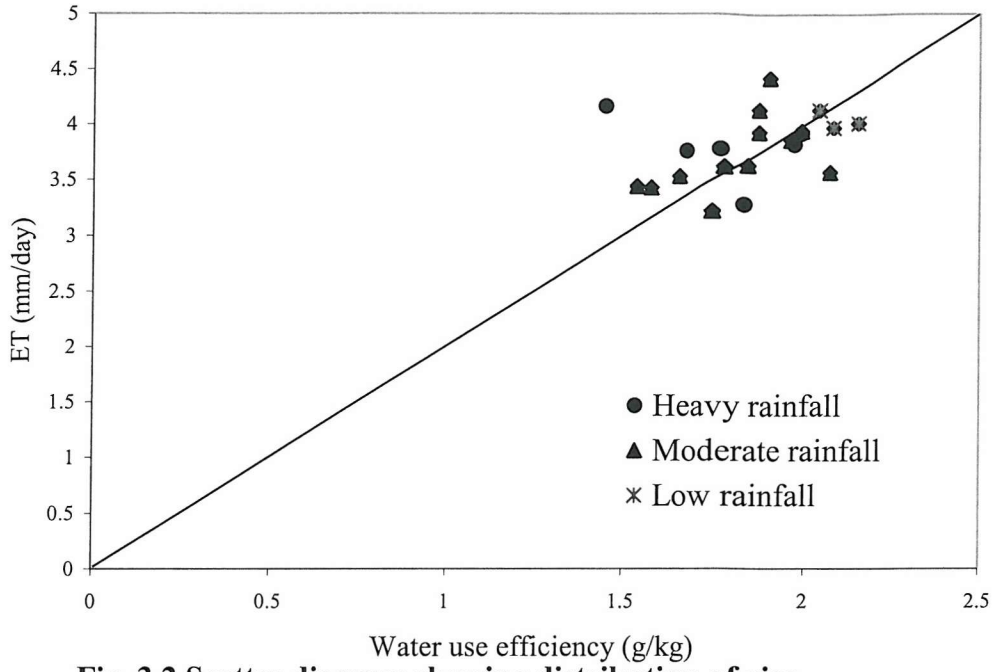

Fig. 3.2 Scatter diagram showing distribution of rice accessions based on ET (mm/day) and water use efficiency (g/kg) in experiment-1

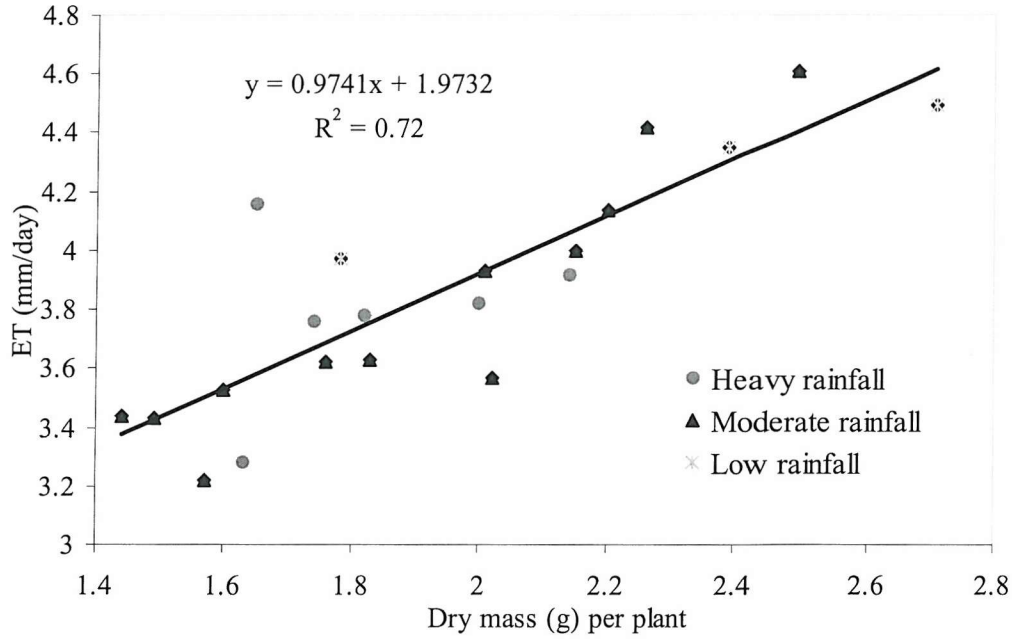

Fig. 3.3 Scatter diagram showing distribution of rice accessions based on ET (mm/day) and dry mass (g) per plant in experiment-1

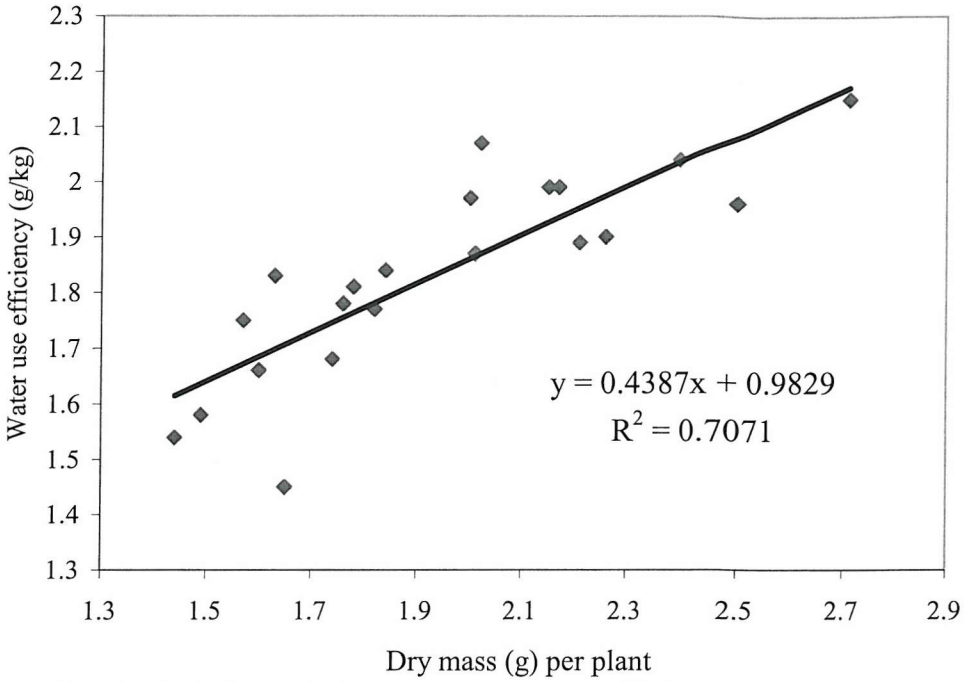

Fig. 3.4 Relationship between water use efficiency (g/kg) and dry mass (g) per plant in experiment-1

A significant correlation existed between drought score and leaf stomata conductance prior to stress (r=-0.30, p=0.006, N=84) (Table 3.4), which indicated that stomata conductance influenced the drought score. The correlation between stomata number and leaf stomata conductance was also significant prior to stress conditions (r=0.62, p<0.001) but this was not significant under stress conditions (Table 3.4). This indicates that stomata conductance is dependent on stomata number prior to stress conditions but not in stress conditions due to their sensitiveness to water.

The correlation between stomata conductance and water use efficiency was significant (r=0.33, p=0.002, N=84) prior to stress (Table 3.4). This showed that water use efficiency was at least partly influenced by stomata conductance. Thus the accessions with favourable leaf characteristics, such as higher stomata conductance prior to stress but lower in stress conditions might be potential source for drought resistance. Surprisingly, correlation was not significant between ET and stomata number, and between ET and stomata conductance.

An attempt has been made to identify the most useful physio-morphological parameters for drought resistance using Principal Component Analysis (PCA) since this reduces the necessary parameters dimensionally whilst keeping the maximum variability in the parameters. The PCA of the physio-morphological characteristics extracted five principal components, which showed a 92.0% variability of the total variation of the parameters (Appendix: Table 3.6). About 60% of total variability of the physio-morphological parameters was encountered by only first and second principal components (Appendix: Table 3.6). The points plotted in the scatter diagrams (Fig.3.5) on the basis of the first two, the third and fourth principal components are accessions. The scatter diagrams revealed that the accessions were distributed heterogeneously and could be divided into three possible groups (Fig.3.5). However, some accessions did not fit into any group and their distributions appeared scattered on the diagrams.

In order to identify and classify the accessions into possible groups with similar characteristics for drought resistance, Hierarchical Cluster Analysis (HCA) was carried out using the variability extracted by principal components. Figure 3.6 shows that at least 4 major clusters were formed from the analysed accessions. The number of groups in two analyses (PCA and HCA) is different which might be due to the heterogeneous distribution of the accessions in scatter diagrams as some accessions fell outside the clusters. It is apparent from the dendrogram that most of the favourable accessions for drought resistance were confined to one group, Group I (5 accessions), and this is considered as the most promising drought resistant group in experiment-1.

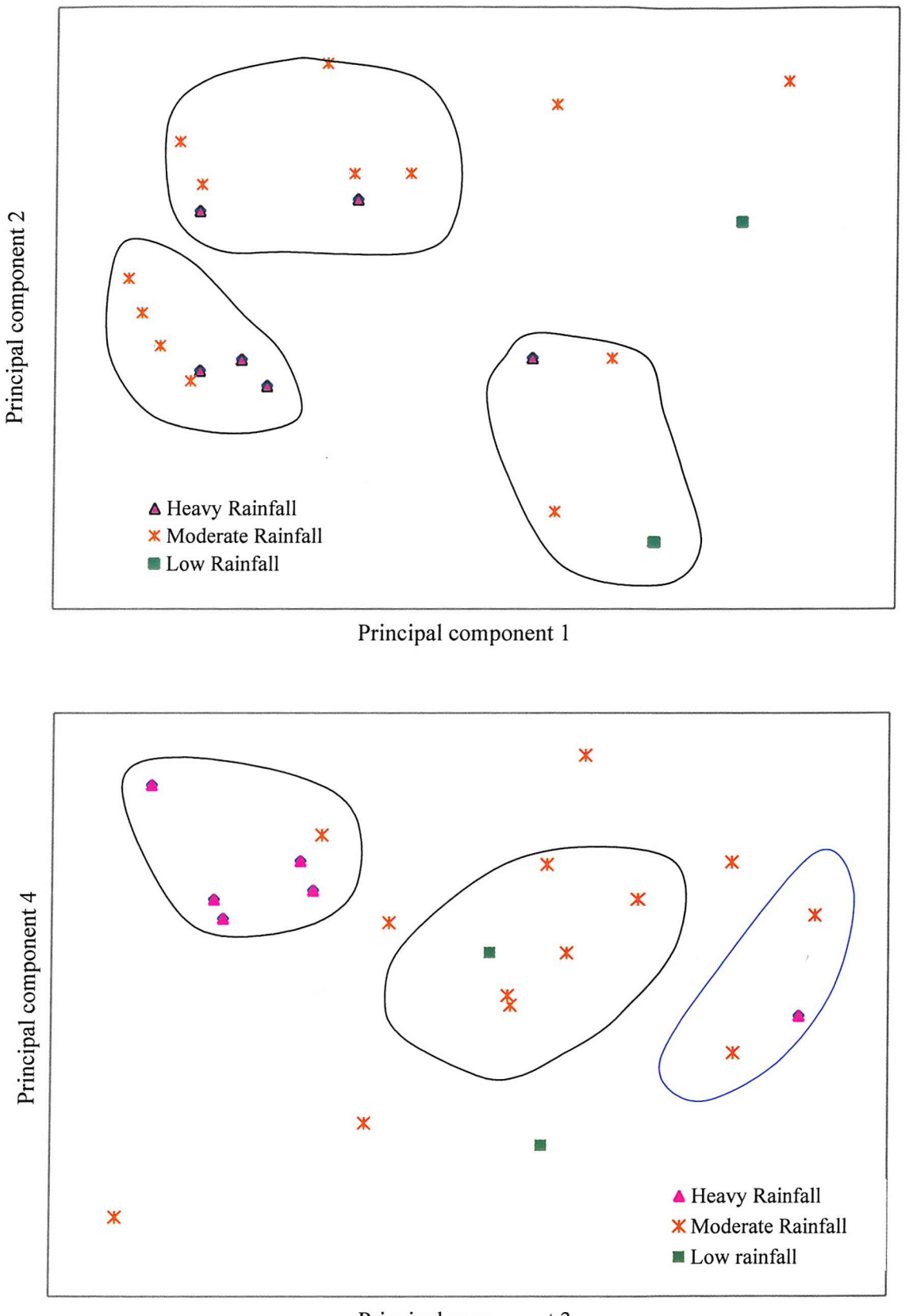

Fig. 3.5 Scatter diagrams showing distribution of rice accessions based on physio-morphological characteristics using PCA in experiment-1

Dendrogram using Ward Method

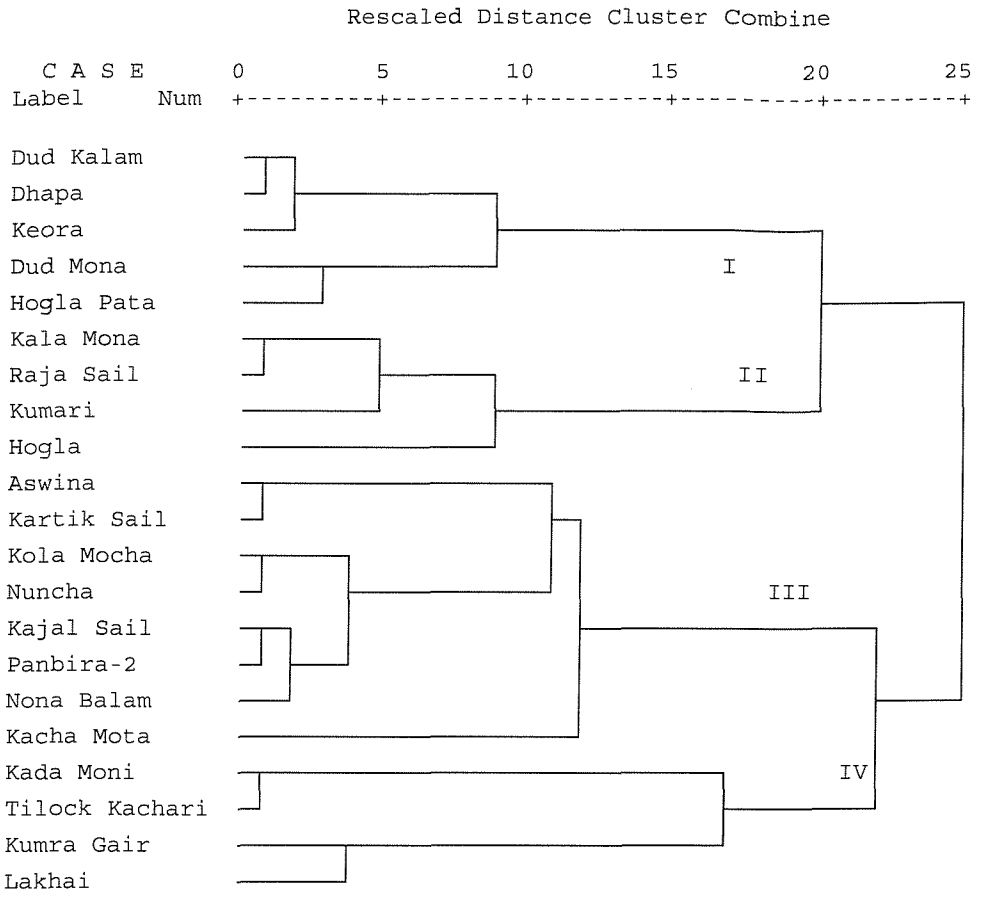

Fig 3.6 Dendrogram showing different groups of rice accessions based on physio-morphological characteristics in experiment-1

The analysis of cluster groups showed that the accessions of a cluster were formed mostly on the basis of ecotypes (Table 3.1 and Fig. 3.6). The effect of the rainfall distribution pattern of the original collection places on the cluster was low (Table 3.1).

Four clusters of different physio-morphological characteristics were compared to justify the validity of cluster groups using one-way analysis of variance (Table 3.5). The results showed that the cluster groups differed significantly from each other in relation to most of the characteristics studied.

Table 3.5 Analysis of variance of different physio-morphological characteristics in 4-clusters in experiment-1

Characteristics	Cluster characteristics				F value	P-value
	Cluster-1	Cluster-2	Cluster-3	Cluster-4		
Shoot length (cm)	55.81	53.50	58.09	54.63	0.81^{NS}	0.662
Days to emergence	11.79	11.38	12.40	10.00	1.23^{NS}	0.273
Tiller number per plant	4.53	6.04	4.99	5.07	1.94^{NS}	0.033
Root length (cm)	36.93	34.36	23.31	26.21	3.49**	0.000
Root dry weight (g)	0.43	0.304	0.28	0.28	1.47^{NS}	0.142
Shoot dry weight (g)	1.26	1.75	1.51	1.49	2.55**	0.004
Total dry weight (g)	1.60	2.21	1.79	1.76	3.72**	0.000
Leaf area cm^2	17.84	18.18	18.29	18.51	0.07^{NS}	0.97
Drought score	1.84	2.53	3.48	5.50	4.57**	0.000
Root-shoot length ratio	0.60	0.64	0.40	0.48	5.42**	0.000
Root-shoot dry weight ratio	0.33	0.29	0.20	0.19	3.34**	0.000
Leaf stomata number	132	145	136	131	1.24^{NS}	0.32
Leaf length (cm)	30.05	36.95	38.45	38.44	35.01**	0.000
Leaf width (cm)	0.72	0.84	0.81	0.77	4.68**	0.000
Stomata conductance (cm/s) prior to stress	0.32	0.36	0.29	0.24	4.81**	0.013
ET (mm/day)	2.94	2.97	3.51	3.59	46.07**	0.000
WUE (g/kg)	1.81	2.08	1.78	1.79	3.69*	0.032
Stomata conductance (cm/s) at stress	0.06	0.047	0.066	0.065	1.92^{NS}	0.16

** Cluster means significantly different from population mean, NS = Cluster means not significantly different from population mean, F value= Fischer significant test value

3.3.2 Experiment-2

In order to verify the results of experiment-1, a second pot screening experiment was carried out using the same and some additional accessions to study their relationship with environmental factors including temperature and solar radiation. It should be mentioned here that ET data was obtained for only four-weeks in experiment-1 whereas in experiment-2 the data was evaluated for the entire observation period. In experiment-2, a total of 51 accessions (including the accessions of experiment-1) were evaluated in the greenhouse to identify drought resistance type(s) using the same parameters as in experiment-1 during the period of May-August 1999. Three accessions, namely: Raja Sail, Kajal Sail and Dud Mona did not germinate and were discarded from the experiment. However, the results showed significant variations of treatment means as calculated F values

(from 1.63 to 16.96) with 47, 141 *d.f.* were greater than tabulated values 1.45 and 1.67 (at 5% and 1% level of significance) in most of the parameters studied (Table 3.6) as seen in experiment-1, which again indicated the variability of the germplasm.

Table 3.6 Descriptive statistics and F values of different physio-morphological characteristics based two ways ANOVA of rice in experiment-2

Characteristics	Minimum	Maximum	Mean ±SE	CV (%)	F Value
Drought score	0.50	7.50	2.87±0.10	31.71	5.86**
Days to emergence	10.00	16.50	12.94±0.12	12.83	1.16^{NS}
Tiller number per plant	3.30	34.00	7.33±0.26	37.62	3.98**
Plant height (cm)	49.70	97.50	71.57±0.59	6.89	7.48**
Root length (cm)	15.50	40.44	25.81±0.66	14.62	4.11**
Root-shoot length ratio	0.21	0.63	0.36±0.01	15.88	4.94**
Stomata conductance (cm/s) prior to stress	0.13	0.54	0.33±0.006	27.53	1.88**
Stomata conductance (cm/s) at stress	0.05	0.11	0.079±0.001	80.50	1.05^{NS}
Stomata number	83	198	131±1.80	19.01	2.91**
Leaf length (cm)	32.50	60.10	45.09±0.38	7.30	6.18**
Leaf width (cm)	0.77	1.60	1.42±0.02	7.69	16.96**
Leaf area (cm^2)	13.47	38.50	22.53±0.48	12.58	9.37**
Root dry weight per plant (g)	0.40	2.80	1.22±0.03	21.16	4.42**
Shoot dry weight per plant (g)	3.20	33.90	6.54±0.11	17.76	3.31**
Total dry mass per plant (g)	3.60	36.30	7.76±0.11	15.66	3.09**
Root-shoot dry weight ratio	0.02	0.40	0.194±0.01	22.35	5.49**
Evapotranspiration (mm/day)	2.66	3.55	3.07±0.01	4.04	1.63*
Water use efficiency (g/kg)	3.41	12.54	6.93±0.10	14.48	2.88**

SE= Standard error of mean, ** Significant at 1% level, *Significant at 5% level, NS = Not significant at 5% level, F value= Fischer significant test value

Similar patterns of results were also observed in respect of visual drought score as seen in experiment-1. The accession Kumra Gair had the highest mean value (mean 6.0 and SE=0.64), which was significantly different from other accessions according to DMRT (Table 3.7 and Appendix: Table 3.7). The accession Kumra Gair had also higher mean value of drought score in experiment-1. Thus the susceptibility of the accession to drought is apparent. The accessions Bina Muri-2, Aug Meghi, Dud Kalam, Agali, Manik Mondal, Huma Gambir, Dhapa, Keora and Kola Mocha had the lowest mean values for visual drought score, ranging from 1.56 to 1.75 (SE=0.13 to 0.48) and DMRT showed similar ranking indicated that

they were not significantly different from each other at 5% level. As mentioned in experiment-1, these accessions had similar characteristics of leaf rolling, and the leaves of these accessions were completely unrolled at morning, rolled at midday, and again unrolled in the late afternoon. However, the accession Hogla and Nona Balam differed in their results from experiment-1. The accession Hogla had a lower mean value (mean = 1.63, SE=0.24) in experiment-1 and higher value (3.25, SE=0.47) in experiment-2, but the accession Nona Balam had a higher mean value (4.33, SE=1.33) in experiment-1 and a lower value (mean 1.88 and SE=0.24) in experiment-2.

Correlation analysis between the characteristics showed some significant positive and negative correlations, as seen in experiment-1 (Table 3.8). Significant correlations were found between drought score and stomata conductance (r=-0.29, p=0.001, N=192), dry root weight (r=-0.26, p=0.000), root length (r=-0.43, p=0.001), ratio of root-shoot length (r=-0.33, p=0.001) and the ratio of root-shoot dry weight (r=-0.28, p=0.000). T-test also showed significant correlation at 5% level of significance between drought score and root length, dry weight of root and root-shoot length ratio. This implied that drought score was mostly related to root length, root-shoot length ratio, and dry weight of root.

Like in experiment-1, the accessions studied showed negative correlation between drought score and dry root weight (r=-0.26, p=0.000, N=192) and root length (r=-0.43, p=0.001, N=192), but correlation was not significant between drought score and shoot length/shoot dry-mass (Table 3.8). This again indicates that root length and dry weight of root were more important for selection than the dry mass of the shoot. Thus the accessions with favourable root characteristics, such as higher dry mass of root and longer root length could be taken as important traits for drought resistance. The root-shoot development systems revealed that accessions Tilock Kachari, Kala Mona, Keora, Kada Moni, Dhapa, Dud Kalam, Dular, Bali Guri and Nona Balam had larger root systems in relation to root length, root dry mass, and ratios of root-shoot length and dry mass (Appendix: Table 3.8), which indicated that these accessions might possess drought resistance characters.

Table 3.7 Summarised mean results of drought related parameters and one standard deviation of promising drought resistance accessions of rice in experiment-2

[a]Name of accessions	Drought score	Leaf stomata number (per sq. mm)	Conductance prior to stress (cm/s)	Conductance (cm/s) at stress	Leaf length (cm)	Leaf width (cm)	Leaf area (per cm^2)	Root dry weight (g) per plant	Shoot dry weight (g) per plant	Root-shoot dry weight ratio	Total dry weight (g) per plant
Dud Kalam	1.56±0.72a	130abc	0.43±0.05l	0.06±0.007	49.42±3.48e	0.87±0.03a	25.46±2.73d	1.51±0.21	6.33±0.39	0.24±0.05	7.83±0.30
Aug Meghi	1.63±0.63a	115ab	0.38±0.02g	0.07±0.02	47.93±1.53e	1.35±0.17e	37.89±4.77k	1.43±0.22	6.10±0.58	0.24±0.06	7.52±0.50
Keora	1.75±0.50a	116ab	0.35±0.03e	0.06±0.005	45.10±2.68c	0.98±0.03b	26.01±2.61d	1.46±0.21	5.78±0.81	0.26±0.05	7.23±0.73
Manik Mondal	1.75±0.50a	115ab	0.37±0.03f	0.07±0.02	41.33±3.46b	1.23±0.13d	29.82±5.38f	1.23±0.10	5.68±0.51	0.22±0.03	6.91±0.48
Humagambir	1.75±0.96a	128bcde	0.37±0.02f	0.07±0.004	45.85±6.84c	1.23±0.19d	33.39±10.55h	1.35±0.10	6.20±0.70	0.16±0.10	7.55±0.69
Agali	1.75±0.50a	134abcd	0.38±0.13g	0.06±0.006	41.78±6.23b	1.13±0.13c	27.62±5.53e	1.24±0.06	6.18±1.04	0.21±0.04	7.42±1.02
Kola Mocha	1.75±0.96a	110a	0.38±0.04g	0.07±0.01	51.43±3.26f	1.00±0.00b	30.13±1.91f	1.45±0.20	6.20±0.68	0.23±0.03	7.65±0.82
Dhapa	1.75±0.25a	120abc	0.37±0.03f	0.06±0.006	43.40±3.49c	1.00±0.10b	25.32±2.38d	1.61±0.12	5.98±0.30	0.27±0.02	7.59±0.37
Bina Muri-2	1.75±0.50a	120abc	0.37±0.10f	0.07±0.008	42.50±3.90 c	1.13±0.05c	28.02±2.81e	1.09±0.15	5.05±0.24	0.22±0.03	6.14±0.35
Kala Mona	1.88±0.75ab	107a	0.38±0.02g	0.07±0.01	57.98±2.19g	1.08±0.05b	36.55±2.77j	1.74±0.14	6.48±0.57	0.27±0.01	8.22±0.70
Kada Moni	1.90±0.49ab	127abc	0.40±0.04h	0.06±0.004	44.60±2.38c	1.25±0.13	32.66±3.61g	1.65±0.17	5.53±0.72	0.28±0.01	7.18±0.80
Bina Muri-1	2.00±0.58	132abcd	0.38±0.05g	0.06±0.007	42.93±2.02c	1.20±0.00d	30.18±1.42f	1.13±0.20	4.98±0.38	0.23±0.04	6.11±0.48
Hogla Pata	2.00±0.41ab	116ab	0.39±0.01g	0.08±0.02	45.25±4.59c	0.90±0.08a	24.03±4.61c	1.20±0.08	5.60±0.52	0.22±0.03	6.80±0.53
Aus Nagra	2.25±0.50ab	142a-e	0.30±0.05d	0.09±0.01	50.95±4.90f	1.55±0.06g	46.36±5.75m	0.80±0.27	7.08±0.94	0.11±0.02	7.88±1.17
Tilock Kachari	2.25±0.50ab	116ab	0.37±0.02f	0.07±0.02	43.50±3.89c	0.85±0.05a	21.68±2.68b	1.69±0.28	6.26±0.66	0.27±0.05	7.95±0.75
Lakhai	2.50±0.82b	143a-e	0.29±0.07d	0.08±0.01	46.40±1.99d	1.10±0.00c	29.91±1.28f	1.28±0.05	4.70±0.76	0.28±0.04	5.98±0.77
Kumari Aus	2.50±1.41b	126abc	0.32±0.03e	0.08±0.01	40.70±2.92b	1.40±0.05f	33.42±3.51h	1.03±0.19	9.53±2.97	0.12±0.03	10.55±2.94
Hanumanjata	2.75±0.96bc	152bcd	0.35±0.07e	0.08±0.01	44.98±1.24c	1.28±0.05d	33.62±1.93h	0.90±0.14	5.45±1.27	0.17±0.01	6.36±1.41
Panbira-2	3.00±0.58c	144a-e	0.26±0.11c	0.09±0.02	46.10±3.07d	0.85±0.05a	22.96±2.28b	1.28±0.29	6.53±1.15	0.20±0.06	7.80±0.97
Bogi	3.00±0.58c	146a-e	0.34±0.12e	0.09±0.01	44.98±2.48c	1.08±0.13b	28.38±2.70e	0.83±0.38	4.80±1.29	0.17±0.04	5.62±1.64
Kacha Mota	4.75±1.25ef	175e	0.35±0.10e	0.095±0.01	50.08±2.11f	0.88±0.05a	25.70±2.27d	1.33±0.46	6.78±0.33	0.19±0.07	8.10±0.67
Kumra Gair	6.00±1.29f	114b	0.18±0.04a	0.10±0.01	48.23±3.63e	1.00±0.00b	28.26±2.12e	0.93±0.17	7.15±1.13	0.13±0.02	8.08±1.18
SE	0.10	2.00	0.01	0.001	0.38	0.02	0.48	0.03	0.11	0.01	0.11
DMRT*	0.29/0.19	5.94/3.93	0.02/0.01	0.002/0.001	1.13/0.75	0.06/0.04	1.43/0.94	0.09/0.06	0.33/0.22	0.03/0.02	0.33/0.22

[a]Accessions in bold letters were also used in experiment-1, SE = Standard error of mean, Mean followed by alphabet showing DMRT ranking: Any two means having a common letter are not significantly different at the 5% level, *Largest/smallest shortage significant range values

Table 3.8 Simple Pearson's correlation coefficient among the drought related parameters in experiment-2

	Drought Score	St. cond. prior to stress	St. conduct at stress	Stomata number per mm^2	Leaf length (cm)	Leaf width (cm)	Leaf area (per cm^2)	Shoot length (cm)	Root length (cm)	Root-shoot length ratio	Shoot dry weight (g)	Root dry weight (g)	Root-shoot dry weight ratio	Total drymass (g)	ET (mm/day)	WUE (g/kg)	Tiller number/ plant	Days to emergence
D. Score	1	-0.27**	0.27**	0.11^{NS}	-0.14^{NS}	-0.02^{NS}	-0.014^{NS}	-0.13^{NS}	-0.43**	-0.33**	0.06^{NS}	-0.26**	-0.28**	-0.01^{NS}	-0.14*	-0.01^{NS}	-0.09^{NS}	-0.25**
		.000	.000	.128	.055	.810	.844	.064	.000	.000	.439	.000	.000	.926	.049	.887	.195	.000
St. conductance prior to stress		1	-0.23**	0.32**	-0.02^{NS}	-0.001^{NS}	0.01^{NS}	-0.03^{NS}	0.25**	0.25**	-0.11^{NS}	0.29**	0.32**	-0.04^{NS}	0.24**	-0.02^{NS}	0.09^{NS}	0.04^{NS}
			.001	.000	.826	.991	.875	.652	.000	.001	.127	.000	.000	.604	.001	.820	.196	.605
St. cond. at stress			1	0.11^{NS}	0.11^{NS}	0.09^{NS}	0.18*	0.07^{NS}	-0.26**	-0.26**	0.23**	-0.24**	-0.36**	0.16*	-0.002^{NS}	0.15^{NS}	0.02^{NS}	-0.03^{NS}
				.124	.129	.211	.014	.359	.000	.000	.002	.001	.000	.029	.984	.043	.744	.651
St. number				1	0.002^{NS}	0.06^{NS}	0.42**	0.02^{NS}	-0.25**	-0.23**	-0.02^{NS}	-0.04^{NS}	-0.02^{NS}	-0.03^{NS}	0.20**	-0.03^{NS}	0.04^{NS}	-0.10^{NS}
					.976	.384	.000	.760	.000	.002	.744	.602	.788	.669	.006	.653	.634	.167
Leaf length					1	0.14^{NS}	0.36**	0.55**	0.07^{NS}	-0.21**	0.14^{NS}	0.04^{NS}	-0.07^{NS}	0.14^{NS}	0.05^{NS}	0.16*	-0.06^{NS}	0.07^{NS}
						.056	.000	.000	.330	.004	.062	.588	.325	.060	.533	.027	.384	.368
Leaf width						1	0.51**	0.48**	-0.14^{NS}	-0.36**	0.15*	-0.23**	-0.25**	0.08^{NS}	0.09^{NS}	0.08^{NS}	-0.12^{NS}	0.07^{NS}
							.000	.000	.083	.000	.045	.001	.000	.251	.190	.267	.103	.339
Leaf area							1	0.45**	-0.17**	-0.38**	0.45**	-0.09^{NS}	-0.33**	0.41**	0.24**	0.41**	0.08^{NS}	0.01^{NS}
								.000	.019	.000	.000	.239	.000	.000	.001	.000	.292	.848
Shoot length (cm)								1	0.07^{NS}	-0.45**	0.23**	-0.02^{NS}	-0.14^{NS}	0.21**	0.17*	0.22**	-0.06^{NS}	0.13
									.320	.000	.001	.826	.056	.003	.018	.003	.385	.068
Root length (cm)									1	0.85**	-0.07^{NS}	0.44**	0.47**	0.04^{NS}	0.02^{NS}	0.06^{NS}	0.14*	0.25**
										.000	.343	.000	.000	.603	.791	.433	.049	.001
R/Shoot length ratio										1	-0.15*	0.41**	0.47**	-0.05^{NS}	-0.04^{NS}	-0.04^{NS}	0.18*	0.18*
											0.04	.000	.000	.497	.576	.604	.011	.013
Shoot dry weight (g)											1	0.14*	-0.45**	0.97**	0.38**	0.94**	0.38**	0.26**
												.047	.000	.000	.000	.000	.000	.000
Root dry weight (g)												1	0.78**	0.37**	0.06^{NS}	0.39**	0.19*	0.10^{NS}
													.000	.000	.410	.000	.009	.161
R/shoot dry weight ratio													1	-0.25**	-0.19**	-0.20**	-0.06^{NS}	-0.05^{NS}
														.001	.007	.005	.437	.479
Total dry weight (g)														1	0.37**	0.97**	0.41**	0.27**
															.000	.000	.000	.000
ET (mm/day)															1	0.24**	0.23**	0.16*
																.001	.001	.026
WUE (g/kg)																1	0.38**	0.25**
																	.000	.000
Tiller number																	1	0.30**
																		.000
Days to emergence																		1

D= Drought, St = Stomata, Cond= conductance, *Significant at P<0.05 and ** Significant at P<0.01, ^{NS} Not Significant at P =0.05 & 0.01, N=192

It appears from the results of experiment-2 that all accessions had higher values of dry matter, shoot length and larger leaf area when compared to experiment-1. This might be due to the longer duration of plant growth and more favourable environmental conditions such as temperature and solar radiation (refer Figs. 3.1a&b) during experiment-2.

Leaf related parameters also showed similar results compared to experiment-1 but leaf length, width and area were higher in experiment-2. Analysis of variance and DMRT showed significant variation between the accessions (Table 3.6 and Appendix: Table 3.9) as observed in experiment-1 indicating the variability of germplasm. Just like in experiment-1, a negative relationship was observed between drought score and conductance prior to stress ($r=-0.27$, $P<0.001$) and this indicated that the accessions, which had higher conductance, resulted in lower drought scores. The accessions Kola Mocha, Kala Mona, Bina Muri-1, Agali, Panbira-1, Tilock Kachari, Keora, Hogla Pata, Kada Moni, Dhapa, Nona Balam, Dud Kalam, Dular, Hashi Kalmi, Manik Mondal, Huma Gambir, Bali Guri, Aug Meghi had the highest mean values for leaf stomata conductance (cm/s), (between 0.37, SE=0.02 and 0.43, SE= 0.025) prior to stress conditions and lower mean values under stress conditions, ranging from 0.06 to 0.07 (cm/s) except Panbira-1 and Dular (Table 3.7 and Appendix: Table 3.9) and these accessions could be considered for drought resistance types.

Covariance analysis using dry mass as the covariate as done in experiment-1 (section 3.3.1) found significant differences in ET (Table 3.6). The points plotted in scatter diagram (Fig. 3.7) are accessions on the basis of ET and water use efficiency showed that the accessions were distributed heterogeneously. It was apparent from Fig. 3.7 that water use efficiency was related to evapotranspiration rate. It was also obvious that some accessions (about 31%) showed higher water use efficiency with lower ET, which were also considered as drought resistant. Moreover, total dry matter production per plant was also linearly related to ET ($R^2= 0.59$) as well as water use efficiency ($R^2=0.97$) (Fig.3.8 and Fig. 3.9), and this was also seen in experiment-1, which indicated that dry mass production was dependent on ET and water use efficiency. It came out from Figs. 3.7 and 3.9 that some accessions could use water more efficiently than others and these could be considered as drought resistant types and at least 15 accessions showed these characteristics.

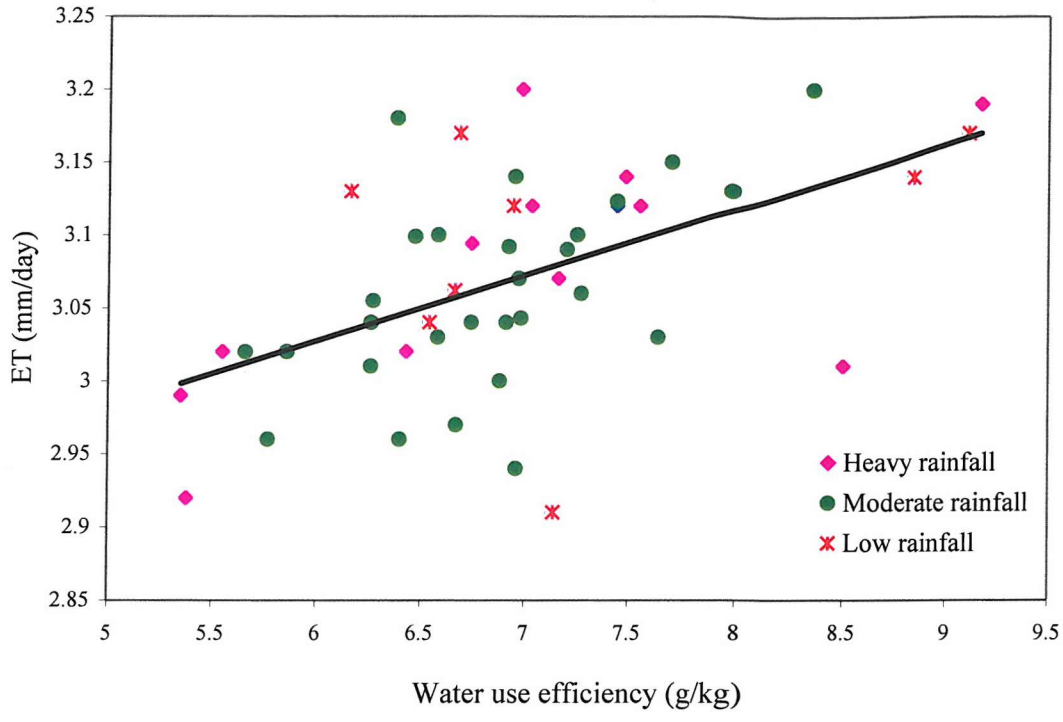

Fig. 3.7 Scatter diagram based on ET (mm/day) and water use efficiency (g/kg) showing distribution of rice accessions in experiment-2

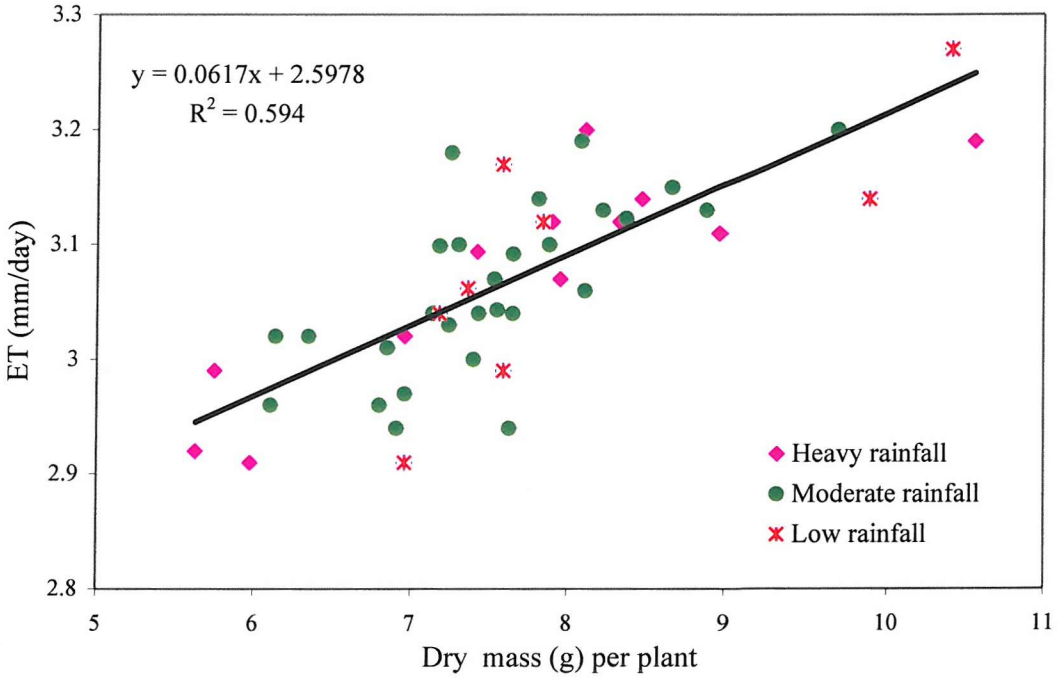

Fig. 3.8 Scatter diagram on the basis of ET (mm/day) and dry mass (g) per plant showing distribution of rice accessions in experiment-2

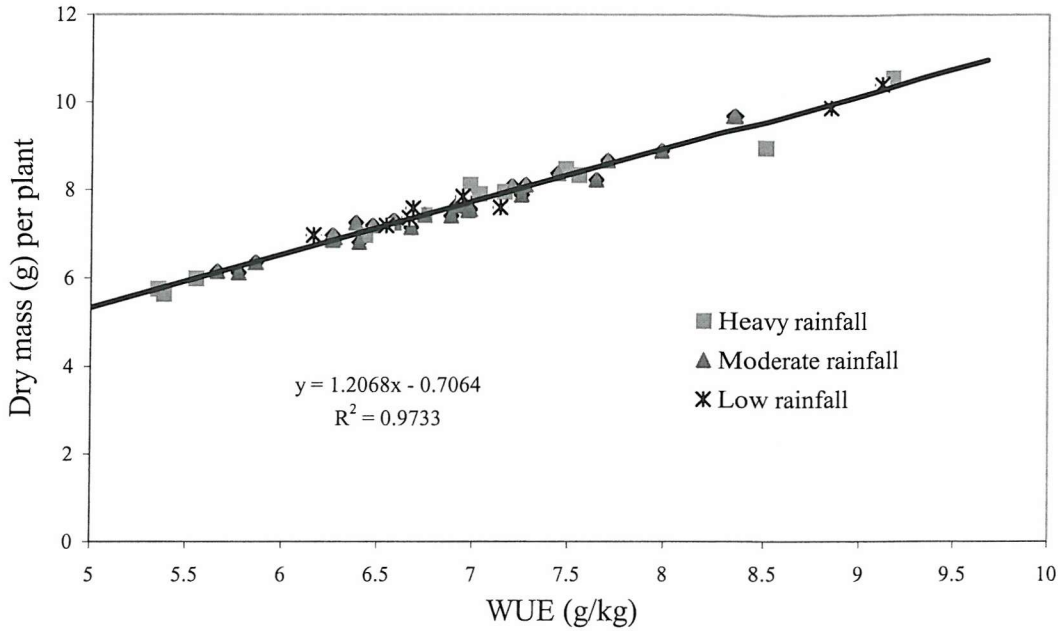

Fig. 3.9 Relationship between water use efficiency (g/kg) and total dry mass (g) per plant in experiment-2

The correlation between total stomata number and stomata conductance prior to stress ($r=0.32$, $p<0.001$) and between stomata conductance prior to stress and evapotranspiration ($r=0.24$, $p=0.001$) were also significant (Table 3.8). Like in experiment-1, total dry mass was also correlated with evapotranspiration ($r=0.37$, $p<0.001$) and water use efficiency ($r=0.97$, $p<0.001$) and between ET and water use efficiency ($r=0.24$, $p=0.001$). As mentioned earlier, this is one of the indications of drought avoidance mechanisms as the plants were able to reduce water loss by the opening and closing of stomata and/or by reducing leaf area during water stress by leaf rolling. Table 3.8 also showed that the correlation between leaf stomata conductance and leaf area was also not significant. This indicated the possibility that the leaf area did not influence the stomata conductance.

An attempt was made to identify the most useful physio-morphological parameters for drought resistance and to classify the accessions into possible groups for identification and selection through Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA). Five principal components of the physio-

morphological characteristics were extracted by PCA, accounting for 82.37% of the whole variation of the parameters (Appendix: Table 3.10). Scatter diagrams on the basis of the first two, third and fourth principal components revealed that the accessions were distributed heterogeneously and could be clustered into four or five possible groups (Fig. 3.10). Like in experiment-1, however, some accessions did not cluster in any group and their distribution appeared scattered on the plane. It also appeared from cluster analysis that at least 6 major cluster groups were formed from the accessions (Fig. 3.11). The number of groups in the two analyses (PCA and HCA) was different, which might be due to the heterogeneous distribution of the accessions in scatter diagrams since some accessions fell outside the clusters. It should be noted here that the accessions of a cluster were formed mostly on the basis of ecotypes of the accession (Table 3.1). Most of the favourable accessions for drought resistance from experiment-2 were confined to one group in the dendrogram (Fig. 3.11). The promising accessions for drought resistance were confined to Group VI in experiment-2 (Fig. 11) and in Group I in experiment-1.

Six cluster groups of different physio-morphological characteristics were compared to justify the validity of the groupings using one-way analysis of variance (Table 3.9). It appears from the table that there is variability in most of the characteristics studied. The validity of the groupings was justified since significant variations were found between the groups.

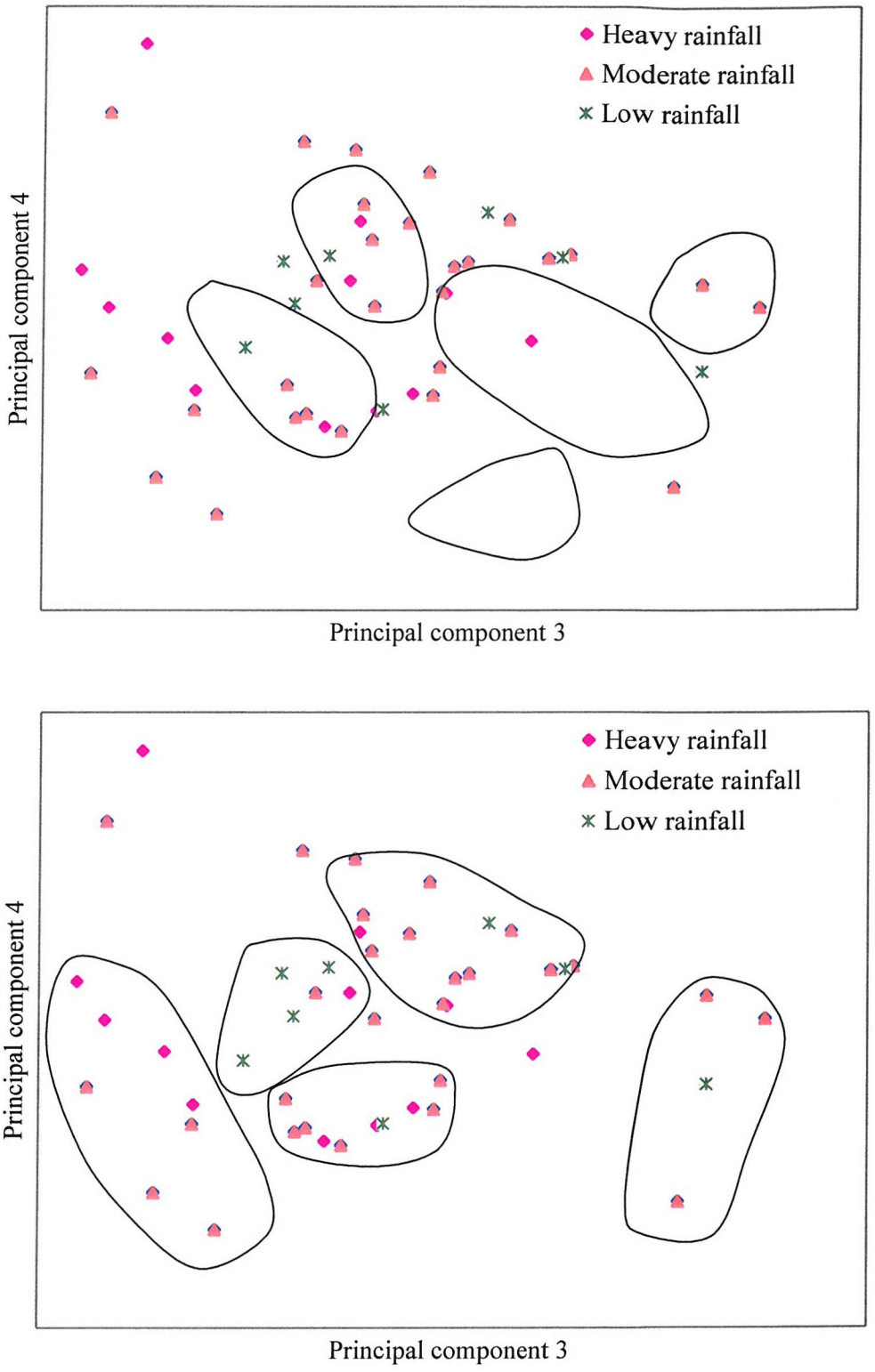

Fig. 3.10 Scatter diagrams showing the distribution of rice accession on the plane defined by principal components 1 &2 and 3 &4 in experiment-2

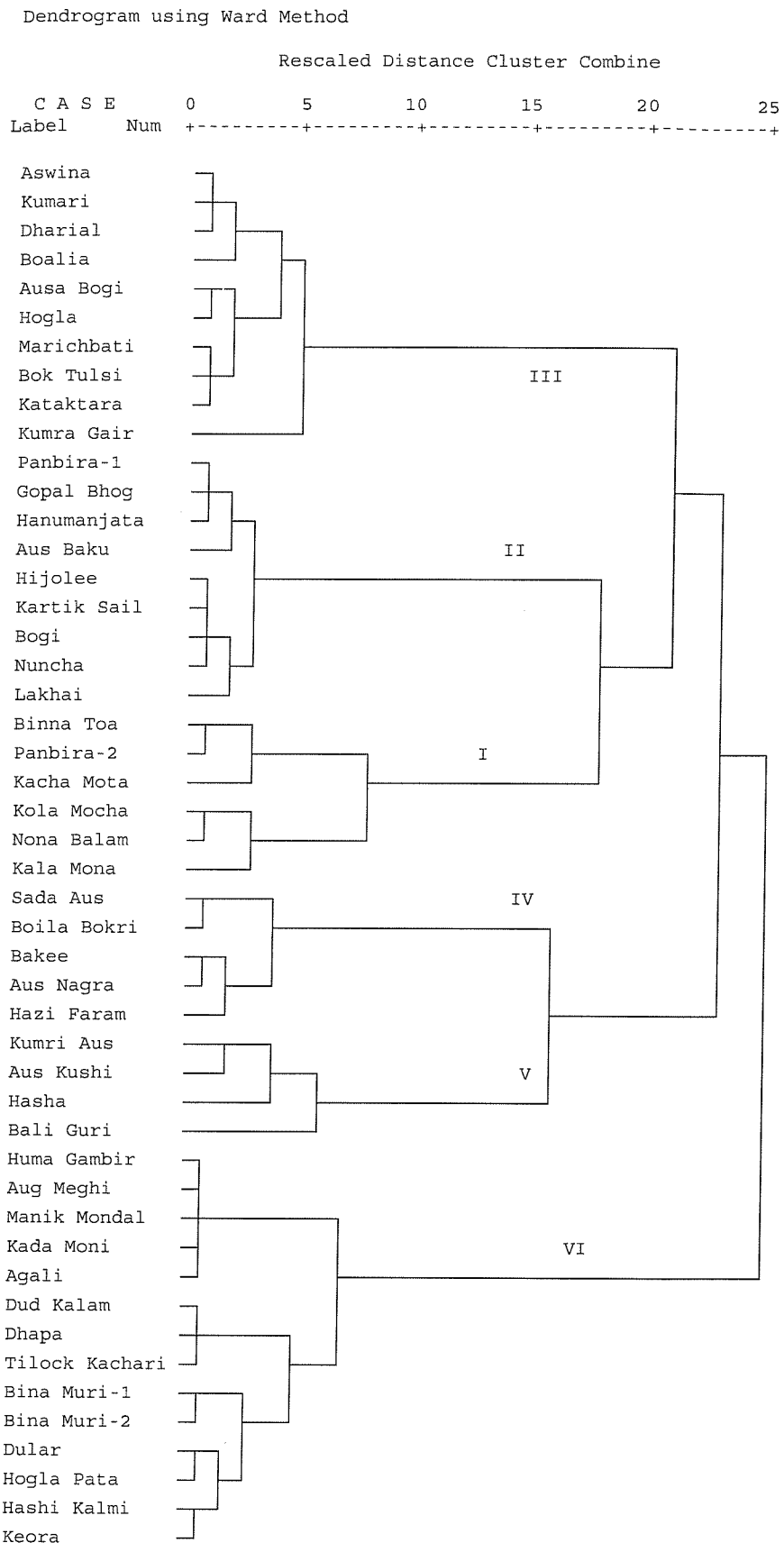

Fig. 3.11 Dendrogram showing different groups of rice accessions based on physio-morphological characteristics in experiment-2

Table 3.9 Analysis of variance of different physio-morphological characteristics in 5-clusters in experiment-2

Characteristics	Cluster characteristics						F-value	p value
	Cluster-1	Cluster-2	Cluster-3	Cluster-4	Cluster-5	Cluster-6		
Drought score	2.75	3.17	4.28	2.55	2.47	1.95	12.05	0.00
Days to emergence	12.21	11.75	12.88	14.45	14.44	13.13	4.56	0.002
Tiller number per plant	7.85	5.65	6.34	7.25	13.36	7.20	9.00	0.000
Shoot length (cm)	72.41	72.17	69.12	82.19	68.70	69.59	4.28	0.003
Root length (cm)	26.25	22.23	23.40	24.29	24.27	30.25	17.56	0.000
Root-shoot length ratio	0.36	0.31	0.34	0.30	0.36	0.44	17.37	0.000
Leaf stomata number	137	148	122	121	135	126	4.09	0.0005
Leaf length (cm)	48.93	45.58	43.71	49.90	40.81	43.61	6.14	0.0002
Leaf width (cm)	0.95	1.19	1.11	1.38	1.23	1.11	4.78	0.0015
Leaf area (cm^2)	20.48	23.54	21.74	30.16	21.99	21.46	6.47	0.0001
Root dry weight (g)	1.53	1.02	1.08	0.95	1.25	1.41	10.98	0.00
Shoot dry weight (g)	6.66	5.75	6.47	7.63	8.24	5.81	12.19	0.00
Total dry weight (g)	8.19	6.77	7.55	8.58	9.48	7.21	9.98	0.00
Root-shoot dry weight ratio	0.23	0.18	0.17	0.13	0.15	0.24	13.27	0.00
ET (mm/day)	3.09	3.07	3.06	3.15	3.16	3.02	5.33	0.000
WUE (g/kg)	7.37	6.16	6.85	7.69	8.38	6.63	9.36	0.000
Stomata conductance prior to stress (cm/s)	0.32	0.34	0.27	0.26	0.34	0.38	9.31	0.000
Stomata conductance at stress (cm/s)	0.08	0.09	0.09	0.09	0.09	0.07	7.65	0.000

** Cluster means significantly different from population mean

The cluster memberships of the rice accessions based on the physio-morphological characteristics in the two experiments were compared to determine suitable accessions for drought resistance. It appears from Table 3.10 that some similarities in cluster memberships in the two experiments occurred, but in the majority of cases the groupings did not correspond between the two experiments. No single group had exactly corresponding members in two experiments. For example, the accessions of group III of experiment-2 were distributed in three different groups of experiment-1. This may be due to different numbers of accessions used in the experiments. However, partial matching showed that the accessions Dud Kalam and Dhapa from experiment-2 corresponded to group I in experiment-1. Similarly, from Group III of experiment-1 three accessions, namely, Panbira-2, Kola Mocha and Nona Balam corresponded to Group I of experiment-2.

Table 3.10 Comparison of distributions of grouping of rice accessions revealed by cluster analyses of two experiments on physio-morphological characteristics

Cluster group in experiment-1 based on morphological data	Name of accession	Cluster group in experiment-2 based on morphological data
No corresponding group*	Binna Toa	I
II	Kala Mona	
III	Kacha Mota, Panbira-2, Kola Mocha, Nona Balam	
No corresponding group*	Panbira-1, Gopal Bhog, Aus Baku, Bogi, Hanumanjata, Hijolee	II
III	Kartik Sail, Nuncha	
IV	Lakhai	
No corresponding group*	Dharial, Boalia, Ausa Bogi, Kataktara, Marichbati, Bok Tulsi	III
II	Kumari, Hogla	
III	Aswina	
IV	Kumara Gair	
No corresponding group*	Hazi Faram, Aus Nagra, Bakee, Sada Aus, Boila Bokri	IV
No corresponding group*	Hasha, Aus Kushi, Kumri Aus, Bali Guri	V
No corresponding group*	Bina Muri-1, Bina Muri-2, Manik Mondal, Huma Gambir, Aug Meghi, Agali, Dular, Hashi Kalmi	VI
I	Dud Kalam, Dhapa, Keora, Hogla Pata	
IV	Tilock Kachari, Kada Moni	
I	Dud Mona	No corresponding group**
II	Raja Sail	
III	Kajal Sail	

*Accession not included in experiment-1 ** Accession not included in experiment-2

3.4 Discussion

The study investigated physio-morphological responses to water deficits between the rice accessions of Bangladesh. The physio-morphological characteristics of different accessions were used for the selection and identification of desired traits. It has been known that drought score, root length, dry root weight, ratio of root-shoot dry weight and length, leaf stomata conductance, stomata number, evapotranspiration and water use efficiency are important parameters for drought resistance studies. These parameters were used in this study and they showed significant variation between accessions with these traits. Several accessions have shown promise for drought resistance and these can be useful in the breeding strategy for rice improvement programmes. The physio-morphological study has shown that 14 out 51 accessions might have traits for drought resistance and suggestions were made for further investigation using biochemical markers.

3.4.1 Comparison with other studies

The visual drought score is one of the best selection indices for drought resistance as this is correlated with root development systems (Ingram, *et al.*, 1990) and leaf water potential (O'Toole and Moya, 1978; Ekanayake, *et al.*, 1985a). In the present study, significant correlation was observed between the visual drought score and the root-related parameters, (such as root length and dry weight of root) as well as between drought score and leaf stomata conductance (Table 3.4). Thus the drought score is the result of the combined effects of root length, root dry weight, root-shoot dry weight ratio, root-shoot length ratio and stomata conductance. This result has shown a large difference between the accessions that were evaluated in this study.

Correlation relationships between dry weight of root and root length and between drought score and dry weight of root and root length have indicated that deep and more extensive root systems caused lower values for drought score. This may be due to enhanced uptake of soil water and as a result plants remained green. In general, plants that transpire more water during their life cycle tend to have better

performance if other conditions such as nutrients and climate are equal (Ludlow and Muchow, 1990;Turner, *et al.*, 2001; Azam-Ali and Squire, 2002). The differences between the cultivars are in the depth and the extent of root per unit shoot weight, which explains the capacity of the cultivars to extract soil moisture from greater depths in order to maintain higher leaf water potential. According to O'Toole (1982), rice cultivars with large total root dry mass and deep root habits are considered to be drought resistant. This implies that the dimensions of root systems are not significant unless the above ground plant parts are taken into account.

A conventional method of describing the degrees of carbon allocation to the above and below ground plant parts is to use the root/shoot dry weight ratio (Turner *et al.*, 2001). The observations in this study showed that the ratios of root-shoot length and root-shoot dry weight were higher in some accessions (Table 3.3). This indicates that these accessions had the capacity to extract more water by exploring larger volumes of soil through their root systems. In the present investigation, it was found that some accessions had greater root systems and higher root-to-shoot ratios (Table 3.3); thus they might be able to maintain high leaf water potential. In experiment-1 at least 7 accessions showed the above-mentioned criteria and may thus be selected for drought resistance. It has also been reported by Chang *et al.*, (1974) that root development systems, especially with regard to the root-to-shoot ratios, are highly correlated with visual field scoring of drought in rice. They suggested that the high correlation between visual drought scoring and maintenance of high leaf water potential is in part attributable to the deep root system profile of traditional upland rice cultivars.

In addition to root related parameters, drought score is also related to leaf related parameters such as stomata conductance. It has been reported by several authors that leaf stomata conductance can be used to measure the opening and closing of stomata since it decreases (conductance) with water stress (Mansfield and Davies, 1981; Collinson *et al.*, 1997; Clifford *et al.*, 2000). This is also in agreement with the findings of the present study, where leaf stomata conductances was found to be decreased to 27.9% in water stress conditions (Table 3.3 and Appendix: Table 3.11). It is apparent that stomata plays an important role in avoiding water loss due

to their sensitivity to water stress, by closing stomata and reducing stomata conductance (Ludlow and Muchow, 1990; Collinson *et al.*, 1997; Azam-Ali and Squire, 2002). This in turn increases water use efficiency when water is limited (Clover *et al.*, 2001). Stomata also have attractive features since they control water loss when water stress occurs and this can be reversed when the stress is removed (Turner *et al.*, 1986a).

In this study leaf rolling, which is one of the water stress symptoms was used in the visual drought score system. The leaf rolling decreases transpiration from leaves by reducing leaf area, and, along with stomata closure, may also contribute to genetical differences in maintaining high water status during water stress conditions (O'Toole and Maguling, 1981). It has been reported by Hopkins (1995) that excessive transpiration is harmful for crop plants because it leads to significant reductions in productivity especially under water-limited conditions. So the crop must keep a careful balance between water uptake and loss to avoid excessive water deficit in the tissues; otherwise it has to pay a yield penalty. This water balance may be achieved by a combination of reduced branching, leaf number, decreased leaf expansion and/or leaf rolling (Clifford *et al.*, 2000). In reality, leaf rolling reduces the leaf area and hence transpiration. Therefore this reduction is beneficial to a plant under water limiting conditions. Moreover, leaf rolling helps to reduce the radiation incident on leaves and consequently reduces leaf temperature and water loss, which leads to increase the avoidance of dehydration. In addition, there would be no yield penalty, as leaf rolling does not occur in the absence of water stress. It should therefore contribute to the stability of yield in the environments with water stress, because it is reversible, and light interception returns to normal after relief of water stress (Turner *et al.*, 1986).

Furthermore, evapotranspiration (ET) also influenced the drought score. In the current study, it was significantly correlated with leaf area, shoot and total biomass in experiment-1 (Table 3.4). It appears from the relationship between ET and dry mass and between ET and water use efficiency that dry mass and water use efficiency increased with the increase of ET. This shows that dry matter production is the consequence of evapotranspiration. However, as mentioned earlier, in some accessions water use efficiency was higher even when their ET

was lower as their dry mass was higher (Table 3.3; Figs. 3.2 and 3.3). This means that these accessions have some capacity to increase water use efficiency, which is believed to be a genetic capacity because drought has minor effect on water use efficiency (Clover *et al.*, 2001).

The study also showed that a small amount of dry matter was produced from one kilogram of water (Table 3.3). It appears from the results that only a small amount of water is retained in the plant compared with water passing through for transpiration. Hopkins (1995) reported that only 5% or less of the total water uptake is actually retained for the growth and development of the plants and even less is required for biochemical activities. This implies that several hundred kilograms (kg) of water is required to produce just one kilogram of dry matter. However, transpiration helps the cooling of the plant, but an essential and inevitable consequence is the diffusion of carbon dioxide for photosynthesis (Azam-Ali and Squire, 2002). Apart from the maintenance of high water content in the tissues, transpiration is also essential for growth and development. Transpiration, as a component of evapotranspiration varies with various factors such as rainfall and its distribution, air temperature, wind and soil type. Generally, evapotranspiration increases when the air is dry, warm and moving. It is therefore important to define the target environment where a potential characteristic may be beneficial for the improvement, adaptation or development of suitable cultivars. In this study, at least five accessions had the highest rates of ET, and the eventual consequence was higher dry matter content.

3.4.2 Comparison of the two experiments

The results of experiment-2 differed from experiment-1 in most cases although treatment deviation patterns were the same as in experiment-1. It appears from the results that all accessions had higher values of shoot- root dry mass, total dry mass, shoot length, leaf length, leaf width, leaf area (Tables 3.3 and 3.8) and water use efficiency compared with experiment-1. It should be mentioned that dry mass and water use efficiency were on average three times higher in experiment-2 than experiment-1 despite the fact that leaf area increased by only 10-20% (Appendix:

Tables 3.3 and 3.9). This might be due to environmental differences such as temperature and solar radiation and the longer duration of experiment-2 (Fig. 3.1a and Fig. 3.1b). Similar results were observed in drought score, root length, stomata number, stomata conductance and ET as seen in experiment-1. In experiment-2, ET was significantly correlated with drought score and stomata conductance prior to stress, stomata number and dry biomass (Table 3.8). Scatter diagrams between ET and water use efficiency and the relationship between ET and dry mass production showed that some accessions had the capacity for higher dry mass production. The water use efficiency of these accessions were also higher (Figs. 3.7 and 3.9). The accessions, which had this capacity, could be considered for drought resistant because their water use efficiency was higher although their ET's were not. In experiment-2 at least 9 accessions showed the highest rates of ET, and the eventual consequence was higher dry matter content.

A significant correlation among the most root-related traits was observed in the study and these traits were interrelated (Tables 3.4 and 3.8). It was observed that the coefficient of determination for water use efficiency (R^2) is 0.87 in experiment-1 and 0.97 in experiment-2 (Appendix: Tables 3.4 and 3.12). The coefficient of determination estimates the proportion of total variation in the dependent variable explained by independent variables. It is likely that more than 87% and 97% of total variation in water use efficiency was due to the combined effect of all variables. Similarly, 98% variation of water use efficiency was due to the combined effect of total dry weight (g) per plant and evapotranspiration when two sets of data were analysed together (Appendix: Table 3.13).

As mentioned earlier, longer root length, greater dry weight of root and higher ratios of root-shoot length and dry weight are believed to play a significant role in drought resistance mechanisms, through the absorption of greater amounts of water from deep soil layers. In experiment-2, no less than twelve accessions had showed the higher values of root-to-shoot ratios and higher values of leaf stomata conductance prior to stress but lower in stress conditions, indicating resistance to drought. The identified accessions might have the capability to maintain high leaf water potential and the mechanism to maintain transpiration through the opening and closing of the stomata. Yoshida and Hasegawa (1982) also reported that rice

accessions with greater values for root systems, especially root to shoot ratios performed better in water limited conditions. The above mentioned accessions also had lower values of visual drought score (Table 3.11), which meant that they could maintain high leaf water potential through opening and closing of stomata. Their leaf stomata conductance (cm/s) also confirmed this because the accessions with lower values of drought score had higher values of conductance prior to stress but considerably lower values in stress conditions.

Principal component and cluster analysis also revealed that most of the possible drought resistant accessions were confined to one group in each experiment (Group I in experiment-1 and Group VI in experiment-2) (Figs. 3.6 and 3.11) although experiment-1 had four groups and experiment-2 showed six groups. This meant that they were similar in relation to their physio-morphological characteristics studied. The higher number of grouping in experiment-2 may be due to larger genetic diversity in the germplasm of experiment-2. Apart from above dissimilarities, it appears from dendrograms (Figs. 3.6 and 3.11) that four out of five potential drought resistant accessions from experiment-1 fell to the same group (Group VI) of experiment-2. This shows their similarity in respect of physio-morphological characteristics such as root length, dry weight of root and shoot, stomata number and conductance. Table 3.11 also showed that the identified accessions had almost similar features in experiment-2 in their key characteristics such as root length, dry weight of root and shoot, stomata number and conductance again indicating their similarity and this could be genetic. It is apparent from the Table 3.11 that only four accessions can be considered possible drought resistance types from the two experiments.

Table 3.11 Selected drought resistant rice accessions with key characteristics

Name of accession	Drought score	Root length (cm)	Shoot length (cm)	Root dry mass (g) per pot	Shoot dry mass (g) per pot	ET (mm/day)	WUE (g/kg)
Dud Kalam*	1.56±0.72	33.58±2.8	63.32±2.6	7.52±1.0	31.62±1.9	3.12±0.1	6.94±0.2
Aug Meghi	1.63±0.63	30.87±3.3	78.43±1.5	7.13±1.1	30.50±2.9	3.07±0.1	6.97±0.5
Keora	1.75±0.50	29.97±1.9	65.89±3.6	7.28±1.0	28.89±4.1	3.03±0.1	6.58±0.7
Manik Mondal	1.75±0.50	30.25±1.9	74.55±3.2	6.16±0.5	28.38±2.5	3.05±0.1	6.27±0.5
Huma Gambir	1.75±0.96	30.42±1.7	76.93±9.3	6.74±0.5	31.00±3.5	3.04±0.1	6.98±1.0
Agali	1.75±0.50	29.08±4.4	79.95±6.4	6.20±0.3	30.91±5.2	3.09±0.2	6.74±0.7
Dhapa	1.75±0.25	34.12±2.3	68.76±2.5	8.06±0.6	29.90±1.5	2.91±0.2	7.14±0.7
Bina Muri-2	1.75±0.50	27.28±4.9	60.90±3.1	5.44±0.8	25.25±1.2	3.02±0.1	5.67±0.2
Kada Moni	1.90±0.49	32.90±1.9	71.75±4.3	8.25±0.9	27.62±3.6	3.04±0.1	6.54±0.8
Bina Muri-1	2.00±0.58	26.55±5.1	61.25±5.4	5.64±1.0	24.88±1.9	2.96±0.1	5.77±0.3
Hogla Pata	2.00±0.41	27.55±2.6	69.20±2.5	6.00±0.4	28.00±2.6	2.96±0.2	6.40±0.3
Tilock Kachari	2.25±0.50	31.22±3.9	61.90±3.5	8.45±1.4	31.27±3.3	3.07±0.1	7.17±0.8

*Accessions in bold letters were selected from both experiments

It can be concluded from the above discussions that environmental factors such as temperature and solar radiation may have influenced the expression of phenotypes. Therefore, selection based solely on the physio-morphological characteristics may give unreliable genetic selections. As genetic selection was the objective of this study, these accessions were further investigated using isozyme systems. This study will provide accurate genetic information on the accessions selected from the physio-morphological investigation, which might have environmental effects.

CHAPTER FOUR

Isozyme study

4.1 Introduction

The genetic diversity of plant populations has traditionally been assessed through phenotypic characters because such traits are easy to observe and measure. In most cases phenotypic characters represent a major genetic diversity. However, the level of genetic diversity resolved solely through physio-morphological characters as observed in the previous chapter is less accurate because of environmental influences on them, especially in the case of qualitative characters.

In recent years, more reliable biochemical markers like isozymes have been used to determine the genetic variability in plant germplasm (Haq, 1996a; Anand, 1998). Markert and Mollar first introduced the term isozyme in 1959 to refer to multiple forms of enzyme proteins produced by living tissues (Paterson *et al.*, 1991). Isozymes are primary products of structural genes; variation in their structure should give reliable information on the variability of the genome itself (Paudyal, 1999) and would be less susceptible to environmental influence.

Isozymes have already been used for the classification of varieties within the *Oryza sativa* (Second, 1982; Glaszmann, 1985; Glaszmann, 1987). It was reported earlier from conventional classification (Chang, 1976; Morishima and Oka, 1981; Oka, 1991) that *Oryza sativa* has two sub-groups; namely: *indica* and *japonica*. Second (1982) also reported the same results (*i.e.* the above sub-groups of *O. sativa)* from his isozyme study. Thus it is apparent that isozyme polymorphism could provide useful information for the classification, selection and identification of germplasm.

Results reported in the previous chapter indicate that environmental factors might have influenced the physio-morphological characters and the selection made from this investigation may be unreliable for gene transfer for drought resistance. Since biochemical markers are more effective for determining the genetic diversity and methods for isozyme analysis are already available for rice, an attempt was made in order to confirm genetic relationships between the accessions included in this study using isozyme systems. This will provide information to make accurate selection, as the selected materials of physio-morphological studies will show similarities to those studied by isozyme systems if these have genetic similarity.

4.2 Materials and Methods

A total of 51 rice accessions were included in the isozyme investigation. The seeds were sown in 15 cm diameter plastic pots having a surface area of 177 cm^2 containing John Innes number 2 compost in a glasshouse at the University of Southampton. The glasshouse temperature was maintained at 27/17±2°C day/night with a day length of approximately 12±1 hours.

Initially three enzyme systems, namely: esterase (EST), alcohol dehydrogenase (ADH) and glutamate oxaloacetate transaminase (GOT) were separated in polyacrylamide gel using a vertical Hoefer SE 600 electrophoresis unit (Hoefer Scientific Instruments, USA). Later five more enzyme systems, viz. catalase (CAT), acid phosphatase (ACP), phosphoglucoisomerase (PGI), malate dehydrogenase (MDH) and peroxidase (PRX) were included in the study. An extraction buffer, gel and a gel running buffer were prepared following the methods of Wendel and Weeden (1990) and IRRI (1995) with some modifications. The extraction buffer consisted of 100 mM Tris-HCL pH 7.5, 5% sucrose (w/v), 5% polyvinylpyrrolidone (PVP-40), 100 mM ascorbic acid, 1% bovine serum albumin and 14 mM (0.1% w/v) mercaptoethanol. Mercaptoethanol was added to the ingredients just prior to use.

Two-week-old fresh leaves (1-2 g) were collected in mesh bags from the glasshouse and kept in a thermoscool box with ice. Five hundred milligrams of leaf samples were transferred to liquid nitrogen for quick freezing. Freeze-dried leaf tissues were ground to a fine powder with 30 mg of insoluble polyvinylpyrrolidone (PVP) using a pre-chilled mortar and a pestle. Five hundred µl of extraction buffer were added to the leaf sample and the sample was further grounded. The enzyme extracts were thereafter centrifuged at 12,000 rpm for 15 minutes and both extracts and other plant samples were kept on ice for use.

Seventy millilitres of 10% polyacrylamide gel was prepared by mixing 24 ml 30% bis-acrylamide, 45 ml gel buffer, 70 µl 14 M mercaptoethanol, 600 µl of 10% ethylene glycol, 100 µl TEMED and 1 ml of freshly prepared 10% ammonium persulphate. The gel buffer (pH 8.3) was composed of either 0.019 M boric acid, 0.004 M lithium hydroxide, 0.047 M Tris-base and 0.007 M citric acid or Trizma base (0.018 g/l) and citric acid (0.0023 g/l). Immediately after mixing the ingredients, 35 ml of gel solution was poured into each gel tray (sandwich assembly) with pre-inserted combs. After gel solidification (about 30 minutes) the combs were removed, creating pre-formed wells. Twenty-five µl of enzyme extracts supernatant was loaded onto the gel well after adding 15 µl loading dye (20% sucrose, 0.05% w/v bromophenol blue).

After loading the samples, they were placed into the gel electrophoretic tank with electrode buffer containing 0.192 M boric acid and 0.038 M lithium hydroxide with a pH of 8.3 or boric acid (0.037 g/l) and sodium hydroxide (0.0048 g/l) pH 8.3 so that the buffer covered the gel completely. Thereafter electrophoresis was done at 60 mA constant current (30mA per gel) with high variable voltage (180 volts at the start, ca 650 at the end of the run as resistance increased) for 4 hours. By this time the dye had crossed the anodic end of the gel. To keep the gels cool, the apparatus was kept on ice (maintaining 4°C) during electrophoresis.

After electrophoresis, the gels were removed from the plates. Subsequently, the gel was rinsed twice with distilled water and soaked in enzyme specific staining solution to detect isozymes. The staining solution for each isozyme is presented in

Table 4.1. The staining procedure was carried out in dark conditions until the bands appeared and accordingly gels were fixed in 50% glycerol for 1 hour to preserve the colour reaction product, localizing the position of isozyme bands. Photographs were taken immediately after fixing the gel, placing the gel on a light box using Canon digital camera (Japan).

4.2.1 Identification of isozyme bands and data analysis

Each band shown by the isozyme was identified by its relative mobility or relative front (rf) with respect to bromophenol blue. The relative mobility was calculated by dividing the distance migrated by the isozyme with that of the marker. The identification of alleles and their assignment to specific loci in some cases were problematic because of the overlapping of loci of EST, PRX and PGI. Due to this overlapping it was difficult to interpret allelic frequency. Therefore the number and relative position of bands were used to determine isozyme phenotypes in each accession and enzyme system following Mouemar and Gasquez (1983). The bands in each enzyme were numbered sequentially starting from the anodal end. Each band was treated as an independent character regardless of its intensity and the accessions were scored with the simple presence (1) or absence (0) for each band as a binary form in all enzymes. Based on the band patterns, the proportion of common fragments between accessions was measured. Cluster analysis was carried out on these scores following the same methods used for physio-morphological characters.

Table 4.1 Staining solutions for different enzymes

Enzyme	Staining solution		Staining time
Glucose-6-phosphate isomerase (GPI) or Phosphoglucoisomerase (PGI) Enzyme Commission Number (E.C. No.): 5.3.1.9	50 mM (.5M) Tris HCl, pH 8.0 NAD or Nicotinamide adenine dinucleotide-phosphate (NADP) Fructose-6-phosphate, sodium salt Glucose-6-phosphate dehydrogenase MTT PMS	50 ml 10 mg 50 mg 10 units 10 mg 2 mg	1 hour
Alcohol dehydrogenase (ADH) E.C. No 1.1.1.1	50 mM Tris HCl, pH 8.0 β-nicotinamide dinucleotide Ethanol MTT PMS	50 ml 10 mg 200 µl 10 mg 2 mg	1 hour
Glutamate oxaloacetate transaminase (GOT) E.C. No 2.6.1.1	Distilled water 0.5 M Tris-buffer α–ketoglutaric acid L-aspartic acid Polyvinylpyrrolidone (PVP-40) Sodium phosphate EDTA Na salt Fast blue BB salt (pH of solution 7.5)	50 ml 20 ml 50 mg 66 mg 25 mg 350 mg 25 mg 50 mg	30 minutes
Esterase (EST) E.C. No 3.1.1. -	100mM Sodium phosphate buffer (pH6.0) α–naphthyle acetate β–naphthyle acetate Fast Garnet GBC salt	50 ml 50 mg 25 mg 40 mg	45 minutes at 40° C
Peroxidase (PRX) E.C. No 1.11.1.7	50 mM Sodium acetate buffer pH 5.0 Hydrogen peroxide, 3% $CaCl_2$ 3-amino-9-ethylcarbazole N,N-dimethylformamide	50 ml 250 µl 50 mg 25 mg 2 ml	1 hour
Acid phosphate (ACP) E.C. No 3.1.3.2	50 mM Sodium acetate buffer pH 5.0 Na α–naphthyl acid phosphate $MgCl_2$ Fast garnet GBG/C salt	50 ml 50 mg 50 mg 50 mg	1 hour
Malate dehydrogenase (MDH) E.C. No. 1.1.1.37	50 mM Tris HCl, pH 8.0 β-nicotinamide dinucleotide Malic acid MTT PMS	50 ml 10 mg 150 mg 10 mg 2 mg	30 minutes
Catalase (CAT) E.C. 1.11.1.6	Hydrogen peroxide 0.01% Water Ferric chloride Potassium ferricyanide	50 ml 50 ml 500 mg 500 mg	1 hour

4.3 Results

The results for isozyme polymorphism are presented below. Since the isozymes ADH and MDH showed fewer bands due to their poor activity and CAT did not show any reaction, these enzymes were excluded from this study.

4.3.1 Acid phosphatase (ACP, E.C. 3.1.3.2)

Ten bands were detected at different relative front (rf) of the bands from the analysis of 51 accessions using the Acid phosphatase enzyme system (Fig. 4.1). The number of bands varied from two to four per accession. The relative front (rf) of the bands varied from 0.81 to 0.12 rf and there were three regions resolved actively and taken as putative loci. Locus Acp-1 was apparently monomorphic whereas loci Acp-2 and Acp-3 were polymorphic (Fig. 4.1). The distribution and relative position of bands gave 9 phenotypes (Fig. 4.1 and Plate 4.1). The accessions analysed were found to be distributed in different ACP phenotypes as follows: 13 (25.49%) in ACP-2, 8 (15.69%) in ACP-3, 7 (13.73%) in ACP-4, 5 (9.80%) in ACP-5, and 4 of each were in ACP-7, 8 and 9 respectively (Table 4.2). The accessions identified as the promising drought resistant types from physio-morphological studies were grouped into the following phenotypes (Table 4.3): ACP-9 (Kada Moni), ACP-7 (Dhapa, Dud Kalam, Hogla Pata, Dud Mona), ACP-6 (Agali, Aug Meghi), ACP-5 (Keora), ACP-4 (Bina Muri-1), ACP-3 (Huma Gambir, Bina Muri-2) and ACP-2 (Dular, Manik Mondal, Tilock Kachari).

4.3.2 Esterase (EST, E.C. 3.1.1.**)

Thirty bands were observed using this enzyme system for accessions studied. The number of bands varied from 3 to 8 per accession at different rf values ranging from 0.96 to 0.11. Five putative loci could be detected when all banding patterns were pooled together. However, bands could not easily be interpreted as the products of particular loci or alleles as they were overlapped. Due to this

difficulty, loci could not be interpreted and this was observed in almost all enzymes studied except for ACP and GOT. Most accessions showed a common band at the position of 0.545 rf followed by 0.36 and 0.27 rf. The number and the distribution of bands according to their relative positions gave 15 isozyme phenotypes (Fig. 4.2).

The accessions were found to be distributed among the phenotypes in almost equal frequencies and the phenotypes EST-1, EST-2, EST-6 and EST-12 were noted to be the most frequent types. However, the accessions identified as the potential drought resistant type from physio-morphological parameters were grouped into different EST phenotypes and the maximum number (3) fell in the phenotype EST-7 (Table 4.3).

Table 4.2 Rice accessions showing (%) different isozyme phenotypes of ACP, EST, GOT, PGI and PRX

Enzyme/phenotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
ACP	9.8 (5)	25.5 (13)	15.7 (8)	13.7 (7)	5.9 (3)	5.9 (3)	7.8 (4)	7.8 (4)	7.8 (4)						
EST	9.8 (5)	9.8 (5)	7.8 (4)	3.9 (2)	5.9 (3)	9.8 (5)	7.8 (4)	5.9 (3)	3.9 (2)	7.8 (4)	7.8 (4)	9.8 (5)	3.9 (2)	3.9 (2)	1.9 (1)
GOT	9.8 (5)	37.3 (19)	1.9 (1)	7.8 (3)	3.9 (2)	5.9 (3)	9.8 (5)	1.9 (1)	23.5 (12)						
PGI	15.7 (8)	11.8 (6)	9.8 (5)	5.9 (3)	13.7 (7)	7.8 (4)	5.9 (3)	5.9 (3)	7.8 (4)	5.9 (3)	5.9 (3)	3.9 (2)			
PRX	7.8 (4)	9.8 (5)	13.7 (7)	7.8 (4)	7.8 (4)	5.9 (3)	1.9 (1)	13.7 (7)	5.9 (3)	7.8 (4)	5.9 (3)	7.8 (4)	3.9 (2)		

(Parenthesis showing number of accession in enzyme phenotypes)

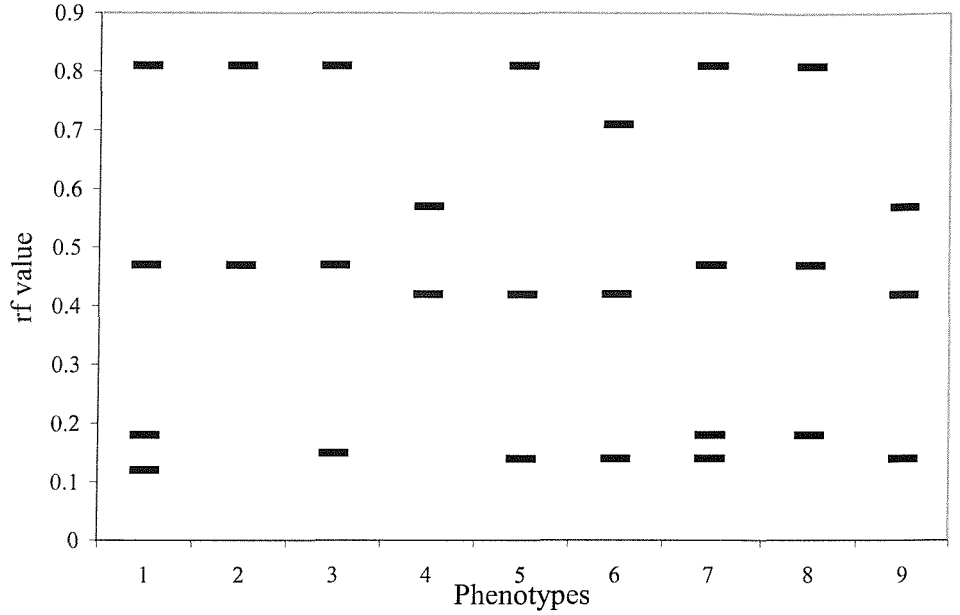

Fig. 4.1 Electrophoretic patterns of ACP

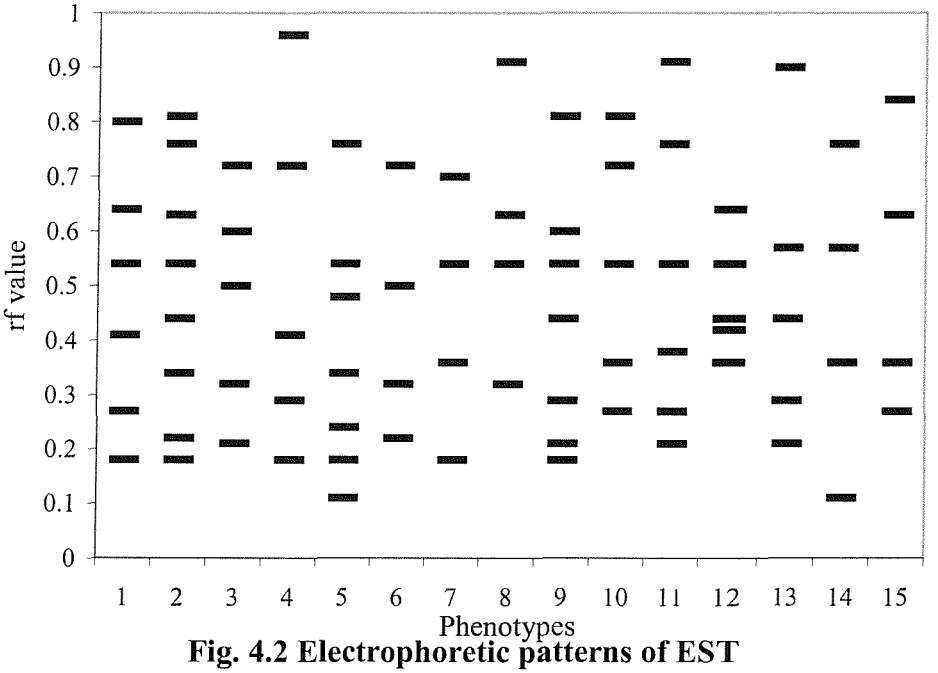

Fig. 4.2 Electrophoretic patterns of EST

Table 4.3 Observed isozyme phenotypes in rice and their frequencies according to ecosystems

Phenotype	Low rainfall	Moderate rainfall	Heavy rainfall	Total	Accession
EST1	0	5	0	5	Dharial, Marichbati, Dular, Kataktara, Hashi Kalmi
EST2	1	4	0	5	Hijolee, Hanumanjata, Hasha, Manik Mondal, Huma Gambir
EST3	2	2	0	4	Sada Aus, Hazi Faram, Bina Muri-1 &2
EST4	1	1	0	2	Kola Mocha, Keora
EST5	0	1	2	3	Bok Tulsi, Bali Guri, Binna Toa
EST6	1	3	1	5	Nona Balam, Kacha Mota, Kada Moni, Aus Nagra, Bakee
EST7	1	3	0	4	Dud Kalam, Dhapa, Dud Mona, Kala Mona
EST8	0	2	1	3	Panbira-2, Aswina, Kumra Gair
EST9	0	1	1	2	Tilock Kachari, Nuncha
EST10	1	3	0	4	Aus Baku, Boila Bokri, Aug Meghi, Aus Kushi
EST11	0	2	2	4	Hogla Pata, Hogla, Raja Sail, Kajal Sail
EST12	0	1	4	5	Boalia, Ausa Bogi, Bogi, Gopal Bhog, Kumri Aus
EST13	0	1	1	2	Panbira-1, Agali
EST14	0	0	1	2	Kartik Sail, Kumari
EST15	0	0	1	1	Lakhai
GOT1	1	4	0	5	Dharial, Dular, Hashi Kalmi, Kataktara, Kada Moni
GOT2	0	13	5	19	Marichbati, Panbira-1, Aus Baku, Hasha, Manik Mondal, Huma Gambir, Hanumanjata, Boilia, Aus Kushi, Bali Guri, Binna Toa, Bakee, Boila Bokri, Aug Meghi, Bok Tulsi, Ausa Bogi, Bogi, Bina Muri-1, Bina Muri-2
GOT3	0	0	1	1	Agali
GOT4	0	1	2	3	Kumra Gair, Kajal Sail, Raja Sail
GOT5	1	1	0	2	Kola Mocha, Keora
GOT6	1	1	1	3	Hazi Faram, Kumri Aus, Aus Nagra

Continued ...

Table 4.3 (cont.)

Phenotype	Low rainfall	Moderate rainfall	Heavy rainfall	Total	Accession
GOT7	2	3	0	5	Gopal Bhog, Hijolee, Nuncha, Sada Aus, Nona Balam
GOT8	0	1	0	1	Lakhai
GOT9	2	7	3	12	Hogla Pata, Kala Mona, Aswina, Kacha Mota, Kartik Sail, Panbira-2, Dud Kalam, Hogla, Kumari, Dud Mona, Dhapa, Tilock Kachari
PRX1	0	4	0	4	Hashi Kalmi, Kataktara, Hasha, Gopal Bhog
PRX2	0	4	1	5	Dharial, Marichbati, Dular, Manik Mondal, Binna Toa
PRX3	1	5	1	7	Aus Baku, Huma Gambir, Bina Muri-1&2, Bakee, Kada Moni, Hanumanjata
PRX4	2	2	0	4	Kumri Aus, Sada Aus, Hazi Faram, Aus Nagra
PRX5	0	1	3	4	Ausa Bogi, Panbira-1, Bogi, Bali Guri
PRX6	0	2	1	3	Hijolee, Nona Balam, Tilock Kachari
PRX7	0	1	0	1	Nuncha
PRX8	1	3	3	7	Kacha Mota, Raja Sail, Kajal Sail, Kartik Sail, Dud Kalam, Dhapa, Dud Mona
PRX9	0	2	1	3	Aswina, Kumra Gair, Panbir-2
PRX10	0	4	0	4	Kola Mocha, Keora, Lakhai, Kala Mona
PRX11	0	3	0	3	Kumari, Hogla Pata, Hogla
PRX12	0	2	2	4	Boalia, Agali, Boila Bokri, Aug Meghi
PRX13	0	1	1	2	Bok Tulsi, Aus Kushi
PGI1	0	4	4	8	Ausa Bogi, Bogi, Gopal Bhog, Nuncha, Panbira-1, Aus Kushi, Bali Guri, Binna Toa
PGI2	0	6	0	6	Dharial, Kataktara, Dular, Aus Baku, Hasha, Kada Moni
PGI3	0	3	2	5	Aug Meghi, Aus Nagra, Bakee, Kumri Aus, Boila Bokri

Continued ...

Table 4.3 (cont.)

Pheno type	Low rainfall	Moderate rainfall	Heavy rainfall	Total	Accession
PGI4	0	2	1	3	Keora, Kola Mocha, Lakhai
PGI5	2	4	1	7	Marichbati, Hashi Kalmi, Hijolee, Boalia, Sada Aus, Hazi Faram, Bok Tulsi
PGI6	0	3	1	4	Manik Mondal, Huma Gambir, Hanumanjata, Agali
PGI7	0	2	1	3	Bina Muri-1, Bina Muri-2, Tilock Kachari
PGI8	1	1	1	3	Dud Mona, Dhapa , Dud Kalam
PGI9	0	1	3	4	Raja Sail, Kartik Sail, Kajal Sail, Kala Mona
PGI10	1	1	1	3	Aswina, Kumra Gair, Kumari
PGI11	0	3	0	3	Kacha Mota, Hogla Pata, Hogla
PGI12	0	2	0	2	Nona Balam, Panbira-2
ACP1	0	4	1	5	Dharial, Aus Baku, Boila Bokri, Bali Guri, Kacha Mota
ACP2	0	9	4	13	Dular, Hashi Kalmi, Kataktara, Manik Mondal, Bok Tulsi, Aus Kushi, Kala Mona, Panbira-2, Tilock Kachari, Aswina, Hogla, Binna Toa, Kumari
ACP3	0	8	0	8	Marichbati, Panbira-1, Hijolee, Hasha, Huma Gambir, Hanumanjata, Kumra Gair, Bina Muri-2
ACP4	2	3	2	7	Bogi, Kumri Aus, Sada Aus, Hazi Faram, Bina Muri-1, Bakee, Nuncha
ACP5	0	3	0	3	Kola Mocha, Keora, Lakhai
ACP6	0	2	1	3	Agali, Aus Nagra, Aug Meghi
ACP7	1	4	0	5	Dhapa, Dud Mona, Hogla Pata, Dud Kalam, Hogla
ACP8	0	1	3	4	Kajal Sail, Nona Balam, Kartik Sail, Raja Sail, Kumari
ACP9	0	2	2	4	Boalia, Ausa Bogi, Kada Moni, Gopal Bhog, Bogi

4.3.3 Glutamate Oxaloacetate Transaminase (GOT, E.C. 2.6.1.1)

GOT is a functional dimeric enzyme occurring in the cytosol, plastid, mitochondria and/or microbody with 4 isozymes. Fifteen bands were detected in this study according to rf of bands and the number of bands varied from two to five per accession (Fig. 4.3). The relative front of the bands varied from 0.66 to 0.14 rf. The inter-accession frequency of bands was variable. For example, the bands position of rf 0.18 and 0.36 were more frequent when compared to other band's position (Plate 4.3). Three regions were effectively detected as three loci. However, regions two and three appeared to have some overlapping of bands and were not used for interpretation. The number and distribution of the bands gave 9 distinct phenotypes or banding patterns (Fig. 4.3). A total of 19 (37.25%) accessions showed identical phenotypes of GOT-2, and 12 (23.53%) showed GOT-9 in this study. The distribution of the accessions in the remaining seven GOT phenotypes were not as frequent as GOT-2 and GOT-12 and the distribution ranged from 1 (1.92%) to 5 (9.80 %) (Table 4.2). The accessions identified as the potential drought resistant types from physio-morphological studies were grouped into the following phenotypes (Table 4.3): GOT-1 (Dular, Kada Moni), GOT-2 (Huma Gambir, Aug Meghi, Bina Muri-2, Bina Muri-1), GOT-3 (Agali), GOT-5 (Keora) and GOT-9 (Manik Mondal, Tilock Kachari, Dhapa, Dud Kalam, Hogla Pata, Dud Mona).

4.3.4 Phosphoglucose isomerase (PGI, E.C. 5.3.1.9)

Twenty-eight bands were revealed from 51 accessions with different relative fronts ranging from 0.88 to 0.08 rf (Plate 4.4) using PGI enzyme system. The number of bands varied from three to six per accession and had three putative loci with some overlapping and all were polymorphic (Fig. 4.4). The distribution and relative position of the bands gave 12 phenotypes. Apart from phenotypes PGI-1 and PGI-5, the accessions were distributed in equal frequencies, ranging from 2 (3.92%) to 5 (9.80%). The accessions identified as the potential drought resistant type from physio-morphological parameters were grouped into different

PGI phenotypes and the maximum number (3) fell in the phenotype PGI-8 (Table 4.3).

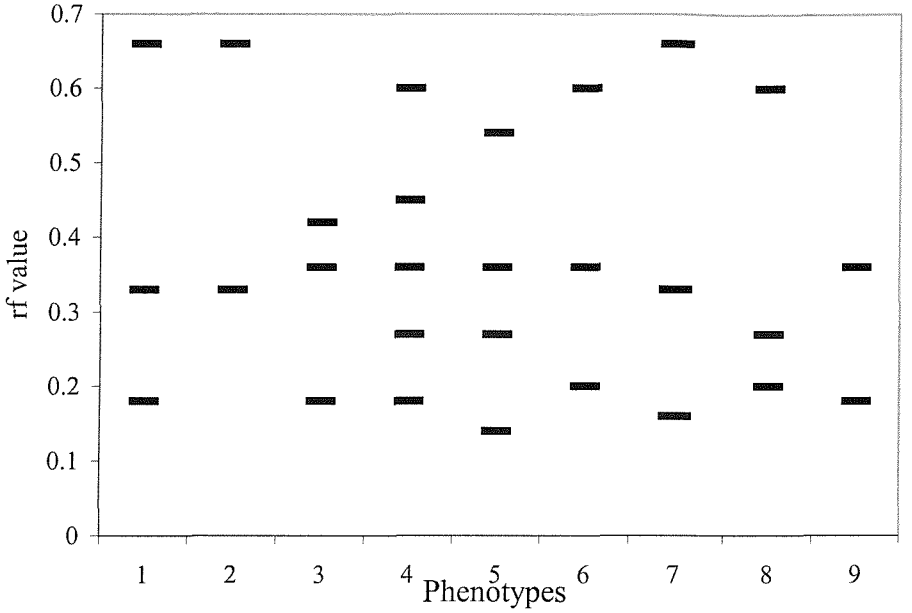

Fig. 4.3 Electrophoretic patterns of GOT

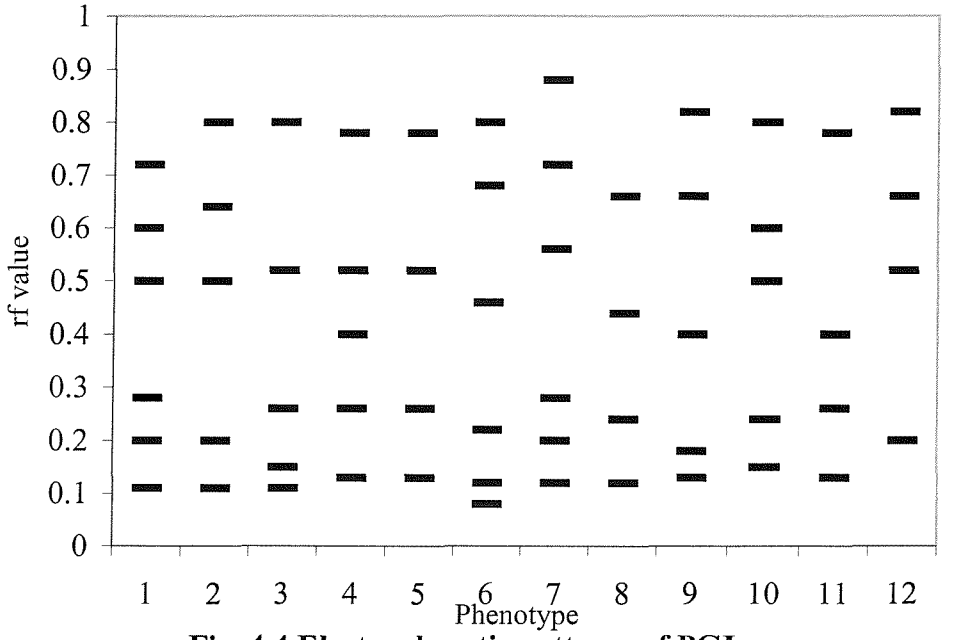

Fig. 4.4 Electrophoretic patterns of PGI

4.3.5 **Peroxidase** (PRX, E.C. 1.11.1.7)

Peroxidase is functionally monomeric and dimeric, occurring in the cytosol and cell wall (Weeden and Wendel, 1990). Eighteen bands were detected in 51 accessions analysed. The number of bands varied from three to six per accession at different rf values between 0.93 and 0.13. Their distribution revealed 13 clear phenotypes (Fig. 4.5). Peroxidase phenotypes PRX-3 and PRX-8 consisted of 7 accessions and the phenotype PRX-7 was found for only one accession. Other phenotypes showed even distribution for accessions ranging from 2 (3.92%) to 5 (9.80%) (Table 4.2). Possible drought resistant accessions from physio-morphological study were found in six peroxidase phenotypes (Table 4.3).

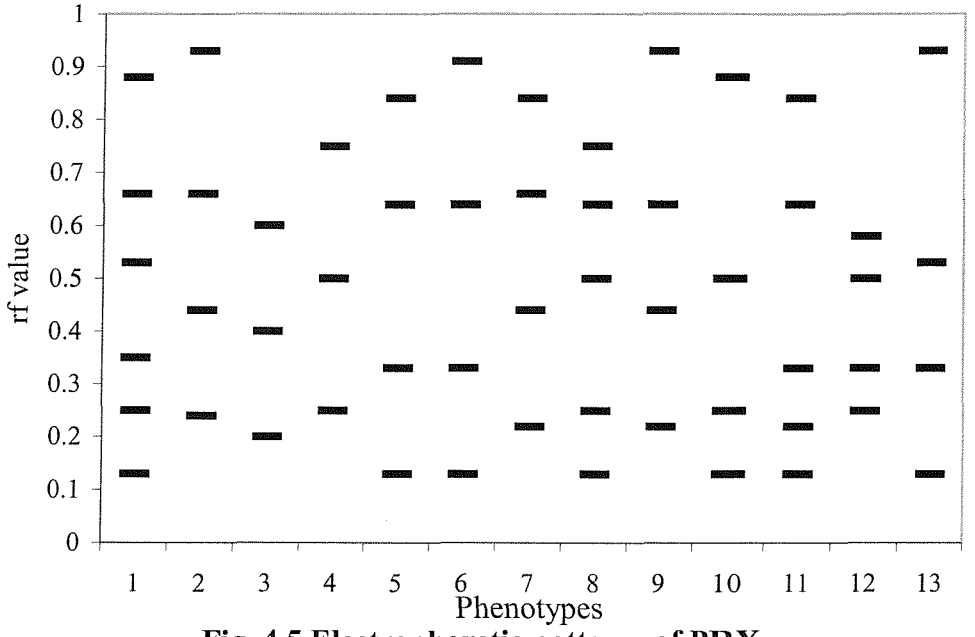

Fig. 4.5 Electrophoretic patterns of PRX

It was apparent from the above results that enzyme system esterase exhibited comparatively higher polymorphism followed by peroxidase (Tables 4.2 and 4.3). On the contrary enzyme systems GOT and ACP displayed the least polymorphism in this study (Plate 4.1 and Plate 4.3).

It appears from Table 4.3 that possible drought resistant accessions were grouped into different isozyme phenotypes. The GOT-9 phenotype possibly includes

maximum number of accessions: Dhapa, Kacha Mota, Dud Kalam, Dud Mona, Tilock Kachari, Hogla Pata, Raja Sail and Hogla. In ACP-7 that was Dhapa, Dud Mona, Hogla Pata, Hogla and Dud Kalam. PRX-8 phenotype consisted of the accessions Kacha Mota, Raja Sail, Dud Kalam, and Dhapa. Phenotypes EST-7 and PGI-8 were composed of three accessions namely, Dhapa, Dud Mona and Dud Kalam. The latter three accessions are included in all other isozyme phenotypes studied and it appears that these can be considered as the most possible drought resistant types from this experiment.

A total of 44 phenotypes were observed whilst 51 accessions were analysed (Table 4.4). It is likely that accessions with similar genetic make-up were grouped together to form a phenotype. Therefore, the accessions, such as Hanumanjata and Huma Gambir might have the same isozyme phenotypes with identical genetic make-up. Similarly, the group Boila Bokri, Hogla Pata and Hogla belonged to same phenotypes with identical genetic make-up.

Table 4.4 Rice accessions showing isozyme phenotypes and their numbers

Phenotypes	EST	GOT	PGI	PRX	ACP	Accession
1	1	1	2	2	1	Dharial, Dular
2	1	1	2	1	2	Kataktara
3	1	1	5	1	2	Hashi Kalmi
4	1	2	5	2	3	Marichbati
5	2	2	6	3	3	Hanumanjata, Huma Gambir
6	2	2	2	1	3	Hasha
7	2	2	6	2	2	Manik Mondal
8	2	7	5	6	3	Hijolee
9	3	7	5	4	4	Sada Aus
10	3	6	5	4	4	Hazi Faram
11	3	2	7	3	4	Bina Muri-1
12	3	2	7	3	3	Bina Muri-2
13	4	5	4	10	5	Kola Mocha, Keora
14	5	2	5	13	2	Bok Tulsi
15	5	2	1	5	1	Bali Guri
16	5	2	1	2	2	Binna Toa
17	6	1	2	3	9	Kada Moni
18	6	7	12	6	8	Nona Balam
19	6	9	11	8	1	Kacha Mota
20	6	6	3	4	6	Aus Nagra
21	6	2	3	3	4	Bakee
22	7	9	8	8	7	Dud Kalam, Dhapa
23	7	9	8	8	7	Dud Mona
24	7	9	9	10	2	Kala Mona
25	8	9	12	9	2	Panbira-2
26	8	9	10	9	2	Aswina
27	8	4	10	9	3	Kumra Gair
28	9	9	7	6	2	Tilock Kachari
29	9	7	1	7	4	Nuncha
30	10	2	2	3	1	Aus Baku
31	10	2	3	12	1	Boila Bokri
32	10	2	3	12	6	Aug Meghi
33	10	2	1	13	2	Aus Kushi
34	11	9	11	11	7	Hogla Pata, Hogla
35	11	4	9	8	8	Raja Sail, Kajal Sail
36	12	2	5	12	9	Boalia
37	12	2	1	5	9	Ausa Bogi, Bogi
38	12	6	3	4	4	Kumri Aus
39	12	7	1	1	9	Gopal Bhog
40	13	2	1	5	3	Panbira-1
41	13	3	6	12	6	Agali
42	14	9	9	8	8	Kartik Sail
43	14	9	10	11	8	Kumari
44	15	8	4	10	5	Lakhai

4.3.6 Cluster analysis of all five enzyme systems

Principal component and hierarchical cluster analysis were carried out, as conducted for physio-morphological characteristics, in order to classify the accessions into different groups according to their genetic make-up and to identify possible drought resistant accessions. During the investigations, all five-enzyme systems were compared to obtain more accurate information for the drought resistance types.

Principal Component Analysis (PCA) extracted 22 principal components, measuring 93.66% of the total variability, and was utilised for cluster analysis (Appendix: Table 4.1). The points plotted below in a scatter diagram (Fig. 4.6a) on the basis of first two principal components are accessions. Scatter diagram showed that accessions could be clustered into five possible groups (Fig. 4.6a). Similarly, the third and fourth principal components revealed four groups of accessions (Fig. 4.6b). It is apparent that the accessions of each cluster were grouped primarily on the basis of ecotype and rainfall distribution pattern (Table 3.1 and Figs. 4.6a&b). However, some accessions could not be classified for any group as shown on the scattered diagram, which might be due to wider genetic distance of those accessions.

Dendrogram (Fig. 4.7) shows 5 main cluster groups of 51 accessions studied. The first group contained maximum number of accessions (16), mainly from rainfed ecosystems and originated from moderate to heavy rainfall areas. This group was divided into three subgroups and the subgroups were joined together according to their similarity. Within each of the three subgroups the determined E value or genetic distance similarity was high. The comparison between subgroup-1 and subgroup-2 showed that similarity was higher where the E values were ≤ 5 and lower where the E values were ≥ 10 respectively.

The second group composed of twelve accessions and divided into two subgroups by cluster analysis, was mostly from upland areas. The origins of

these accessions are mostly within low to moderate rainfall areas. The accessions Hogla Pata and Hogla showed the highest similarity ($E \leq 2$) followed by Hashi Kalmi and Marichbati ($E \leq 4$). All these originated from moderate rainfall areas (Table 3.1).

The third group was formed by only four accessions, which had very low similarity values of $E \geq 10$ compared to other groups.

The fourth group had a very low similarity index where, E values appeared to be ≥ 15 and consisted of eight accessions. These accessions belonged to low to heavy rainfall areas.

The fifth group was formed of eleven accessions, where similarity value varied widely and ranged from $E \leq 1$ to $E \geq 20$. These accessions belonged to different ecosystems.

It was apparent from the analysis of clusters that the accessions varied widely, as their re-scale distance (E) value was wide; from 1 to 24 (Fig. 4.7). The highest similarity of accessions was found in the group III as the E value was less than 5 and the highest dissimilarity was found to be in Group V (Fig. 4.7) where the E value was more than 10. This implies that the genetic similarity of the accessions was high within each group but considerably lower between the groups (except group III), which indicate the presence of genetic variability.

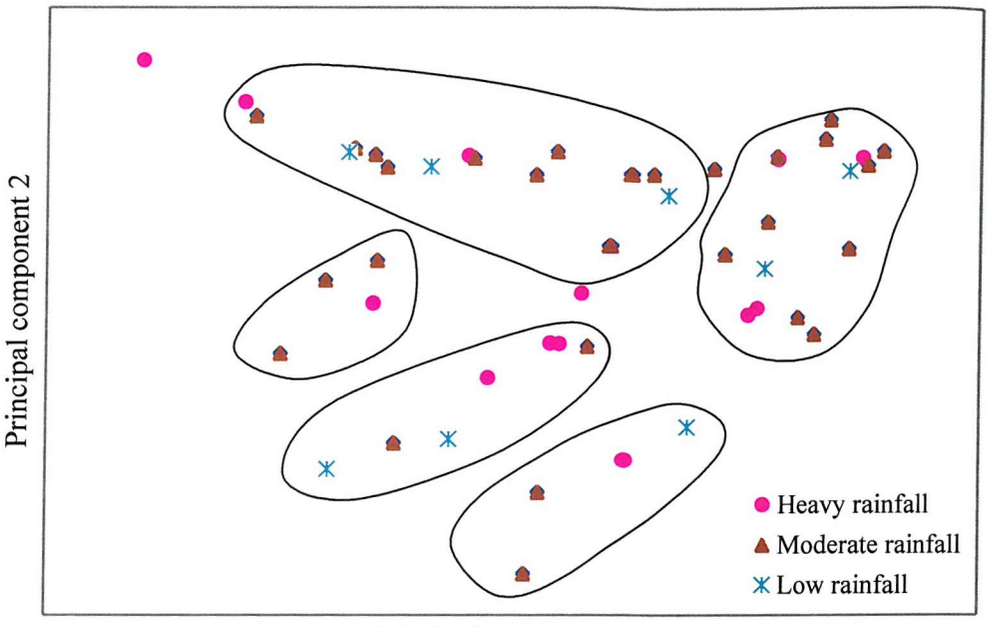

Fig. 4.2a A scatter diagram showing the distribution of rice accessions using principal component 1 and 2

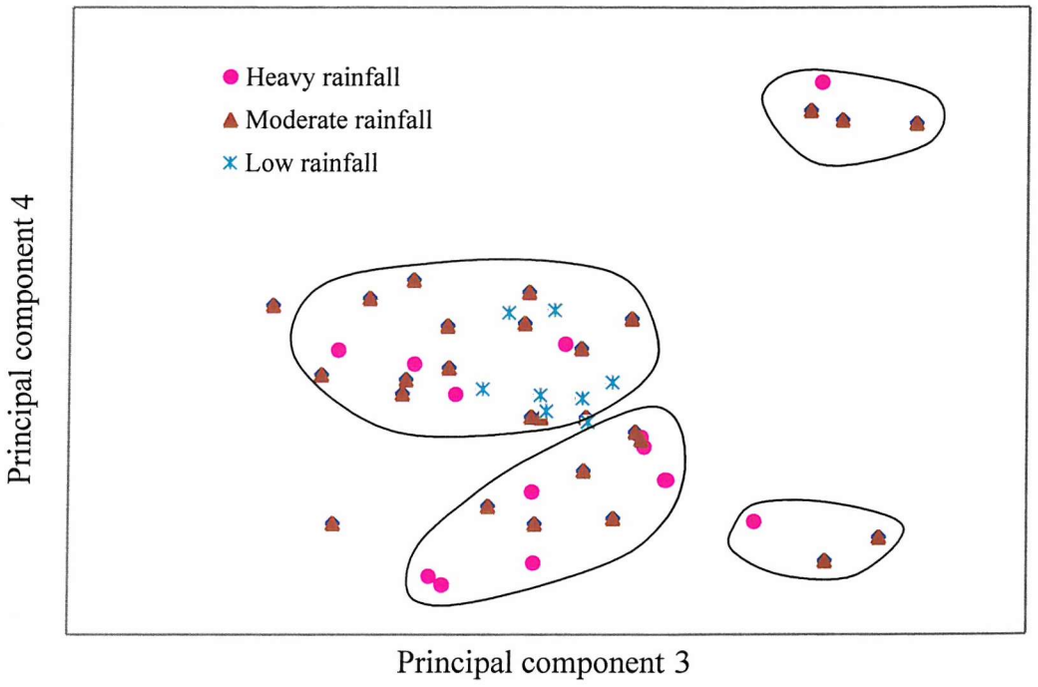

Fig. 4.2b A scatter diagram showing distribution of rice accessions using principal component 3 and 4

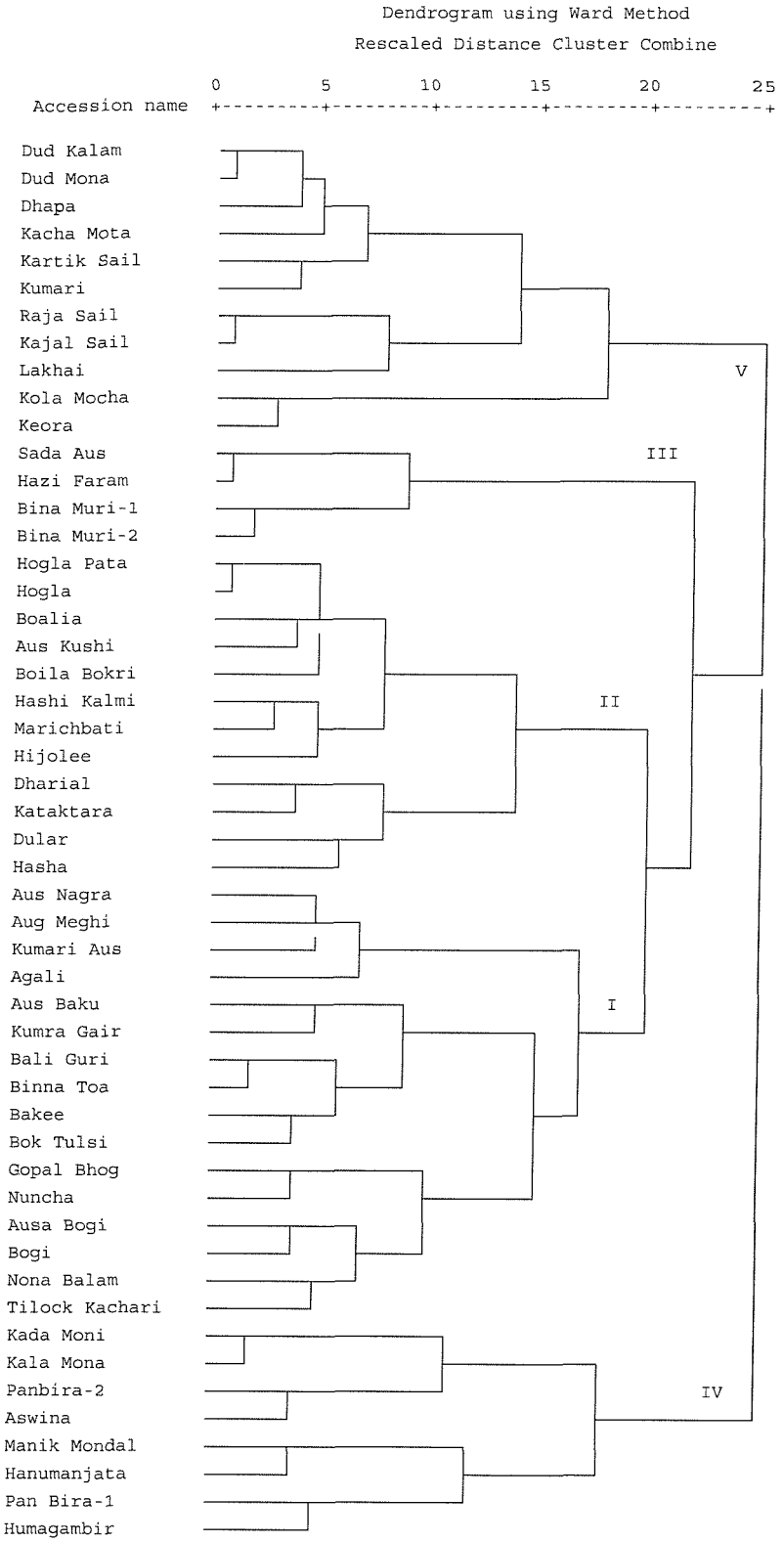

Fig. 4.7 Dendrogram showing different groups of rice accessions based on five isozymes

The cluster memberships of 51 accessions from the two-cluster analysis based on isozyme and physio-morphological characteristics were compared (Table 4.5). In physio-morphological characteristics 4-6 clusters were formed (Figs. 3.8 and 3.16) whereas in isozyme, five major clusters were identified (Fig. 4.7). There was some synchronization observed in two analyses for memberships but in most cases the groupings of accessions did not match with isozyme and physio-morphological data. The majority members of the physio-morphological groups grouped into different clusters of isozymes. For example, the accessions of the physio-morphological group I in experiment-2 were distributed into three isozyme groups (I, II and IV), with the majority of the accessions falling into isozyme group I. Similar patterns were also observed in other groups when both physio-morphological and isozyme studies were considered. According to these studies, the accessions Dharial, Dular, Hashi Kalmi, Kataktara, Marichbati and Hijolee seemed to be genetically similar (Table 4.5) and were included in isozyme group-I. It is apparent from Table 4.5 that only two accessions, namely: Dhapa and Dud Kalam have consistently matched with the results of the physio-morphological investigations, and could be considered possible drought resistant types.

Table 4.5 Comparison of grouping distribution of rice accessions revealed by cluster analysis of isozyme and physio-morphological characteristics

Cluster physio-morphological data in experiment-1	Cluster physio-morphological data in experiment-2	Name of accession	Cluster group of isozyme analysis
No corresponding group*	I	Binna Toa	I
II		Kala Mona	IV
III		Panbira-2	IV
III		Kola Mocha, Kacha Mota	V
III		Nona Balam	I
No corresponding group*	II	Panbira-1, Hanumanjata	IV
		Bogi, Gopal Bhog, Aus Baku, Hijolee	I II
III		Kartik Sail	V
III		Nuncha	I
IV		Lakhai	V
No corresponding group*	III	Dharial, Kataktara, Marichbati, Boalia	II
		Ausa Bogi, Bok Tulsi	I
II		Kumari	V
II		Hogla	II
III		Aswina	IV
IV		Kumara Gair	I
No corresponding group*	IV	Hazi Faram, Sada Aus	III
		Aus Nagra, Bakee	I
		Boila Bokri	II
No corresponding group*	V	Kumri Aus, Bali Guri	I
		Hasha, Aus Kushi	II
No corresponding group*	VI	Bina Muri-1, Bina Muri-2	III
		Manik Mondal, Huma Gambir	IV
		Aug Meghi, Agali	I
		Dular, Hashi Kalmi	II
I		Dud Kalam, Dhapa, Keora	V
I		Hogla Pata	II
IV		Tilock Kachari	I
IV		Kada Moni	IV
I	No corresponding group**	Dud Mona	V
II		Raja Sail	V
III		Kajal Sail	V

*Accession not included in experiment-1 ** Accession not included in experiment-2

Table 4.6 Most promising rice accessions for drought resistance according to physio-morphological characteristics and isozymes investigation

Experiment-1	Experiment-2	Isozyme study[a]
Dhapa, Dud Mona, Hogla Pata, Dud Kalam, and Keora	Hogla Pata, Agali, Aug Meghi, Humagambir, Bina Muri-1&2, Dhapa, Dud Kalam, Kada Moni, Manik Mondal, Keora, Dular and Tilock Kachari	**Dhapa, Dud Kalam**, Dud Mona, Keora, Kajal Sail, Kacha Mota, Kartik Sail, Kumari, Raja Sail, Lakhai and Kola Mocha

[a]Accessions in bold letters are considered as possible drought resistant types

4.4 Discussion

The results from the uni- and multivariate analysis of physio-morphological characteristics showed that environmental factors may have contributed to the grouping of genotypes used in this study. Hence, an investigation was carried to detect genetic similarities within and between accessions using isozymes systems since they measure the variability more accurately.

Initially a total of eight enzyme systems were included in this study to explore reasonable number of loci to obtain maximum information on genomes to confirm the genetic diversity already recorded in chapter 3. However three enzyme systems such as ADH, MDH and CAT have been excluded from the detail study as ADH showed only one locus, MDH showed very poor activity and CAT did not show any reaction in initial observation. Thus the results discussed here are from the remaining five enzyme systems.

It was apparent from the results that variability existed at molecular level among and between the accessions (Tables 4.2 and 4.3). This was revealed by the number and relative position of enzymatic bands of different enzyme systems. According to the banding patterns of different enzyme systems the accessions were grouped into different phenotypes. In GOT, nine groups or phenotypes were detected which implied that the level of genetic diversity was low. Similar results were also observed in ACP where accessions were grouped into 9

phenotypes and 13 (25.49%) accessions formed an identical phenotype of ACP-2 with only two bands.

In contrast, the accessions were grouped into 15, 13 and 12 distinct phenotypes in respect of isozyme EST, PRX and PGI, which identified them as to be highly polymorphic. The polymorphic nature of EST, PRX and PGI were also shown in Plates 4.2, 4.4 and 4.5. Unlike in ACP and GOT, the accessions were almost evenly distributed among these isozyme phenotypes.

It was apparent from the above results that banding patterns were high in all enzymes systems indicating again the polymorphic characteristics of isozyme systems. This polymorphism may be due to genetical factors rather than environmental because environments generally do not influence the isozyme bands.

It appeared from the results that some of the isozyme patterns occurred commonly in accessions and some of them were rarely found in accessions (Tables 4.3 and 4.4). This indicates that some accessions are genetically more distant than others, which was also revealed by the scatter diagrams (Figs. 4.6a&b).

Since isozymes are primary gene products, variation in their structure could give reliable information on the variability of genomes. The 44 phenotypes noted in 51 accessions may be equated to the genotypes of these accessions indicating genetic variability. The high level of variation in the isozyme's banding patterns, especially those of EST, PER and PGI, have complemented the genetic differences observed on physio-morphological characters. This indicates that the rice accessions obtained from Bangladesh have considerable genetic variation.

Further information came from the cluster analysis where accessions were grouped into five major clusters although more groups were expected (as 44 different isozyme phenotypes were identified). It indicates that a cluster may not necessarily be composed of accessions with identical genetic make-up but may

have been composed because of similar phenotypic expression. Similar results were also reported by other authors (Ardhya *et al.*, 1998; Paudyal, 1999). The grouping patterns of the present study are closer to the results of Glaszmann (1987) where rice accessions were classified into six groups when he used some *japonica* type cultivars in the isozyme study.

Comparisons between physio-morphological and isozyme cluster analyses revealed that the majority of accessions did not indicate similarity, however, several accessions showed drought resistance characteristics and at least two accessions could be selected for drought resistance. The most likely reasons for the lack of similarities between the patterns of clusters in physio-morphological and isozyme studies may be due to the fact that direct gene product, which is less susceptible to environmental changes, and also because of plants grown in an identical environment. The other reason may be that five isozymes were used to assess the genetic differences of the rice accessions and these represented a very small fraction of the total genome in comparison to the amount of genetic material involved in producing the complex morphology of the plant. Variation was also reported in the case of Vietnam upland rice when compared between morphological and microsatellite DNA polymorphism for *japonica* and *indica* type rice (Thanh *et al.*, 1999). Furthermore, variation was reported in wild species of rice from the study of physio-morphology, isozyme and RAPD markers (Suh and Morishima, 1997; Pisupati, 1999). Similar results have also been reported in other crop species. The germplasm of Foxtail millet (*Setaria italica*), which were very similar in their morphological characters, were clearly distinguished by isozyme markers (Wang *et al.*, 1995). The high level of morphological variation associated with a number of *Vigna vexillata* germplasm did not correspond to a high level of allozyme or RAPD polymorphism, but the clusters based on RAPD data were similar to isozyme allelic frequency (Spinosa *et al.*, 1998). Thus it can be assumed that classification based on physio-morphological traits do not always necessarily match the genetic relationships determined by biochemical and molecular markers.

In conclusion, it is apparent from the isozyme study that only two accessions, namely: Dud Kalam and Dhapa showed consistently different isozyme banding patterns. These two accessions were also identified from physio-morphological studied as potential drought resistance types. Therefore the accessions Dud Kalam and Dhapa could be selected (Table 4.6) with reasonable accuracy that these may have drought resistant traits, which could be incorporated in high yielding and good quality cultivars through breeding programmes for the development of drought resistant rice cultivars. This has been attempted through *in vitro* fertilization methods as several researchers have used this method successfully for maize improvement and the work is described in the next chapter.

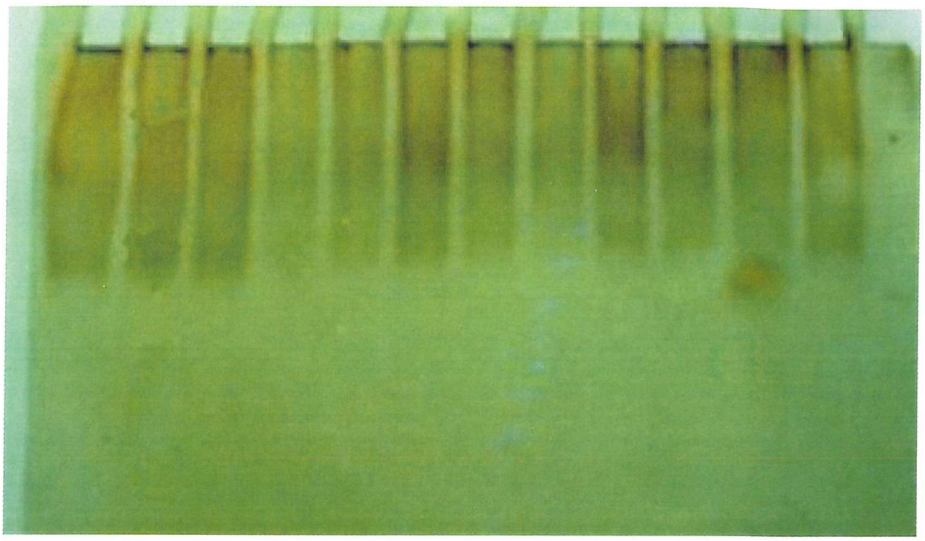

Plate 4.1 Variation in banding patterns of ACP

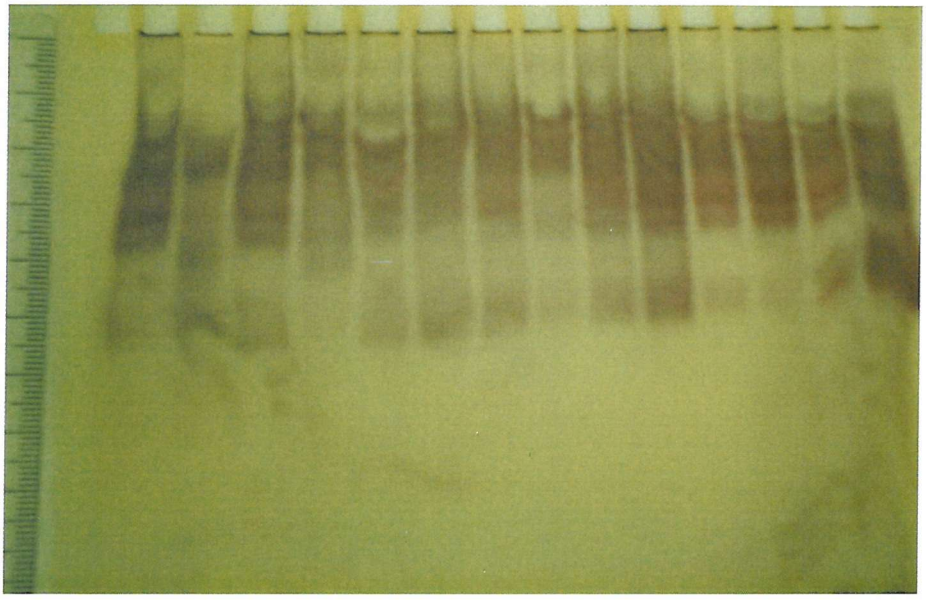

Plate 4.2 Variation in banding patterns of EST

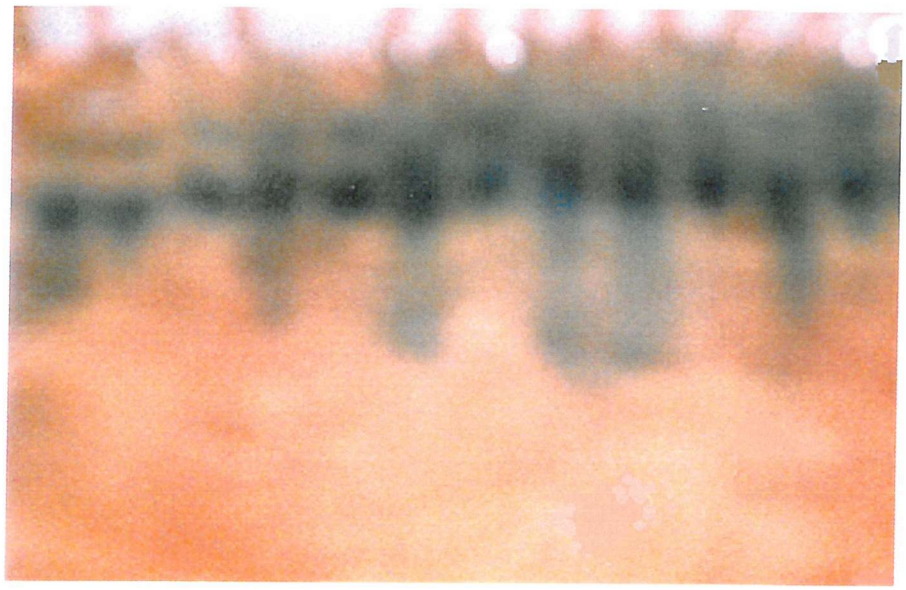

Plate 4.3 Variation in banding patterns of GOT

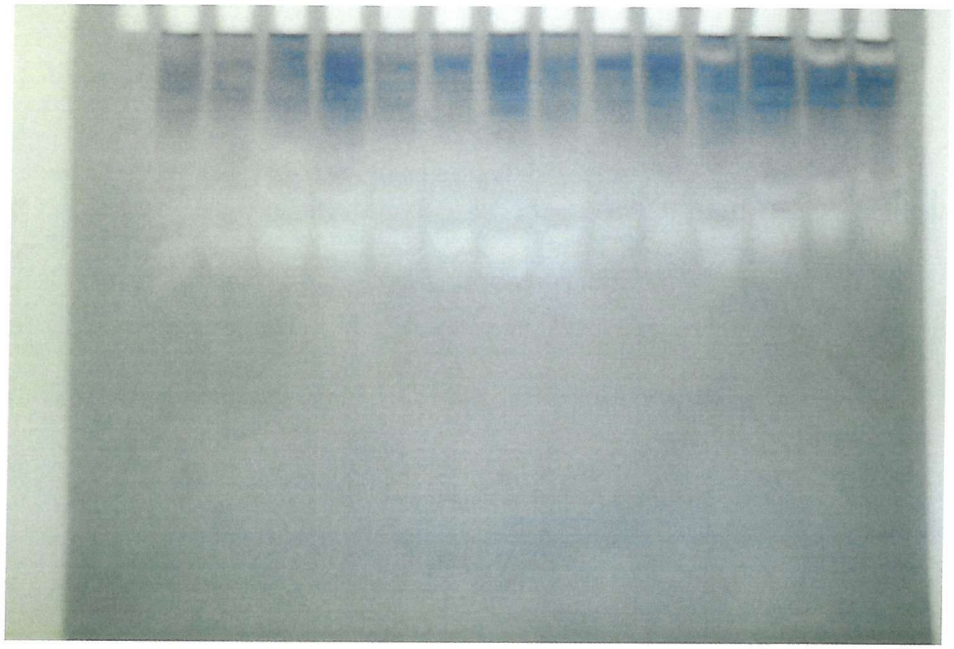

Plate 4.4 Variation in banding patterns of PGI

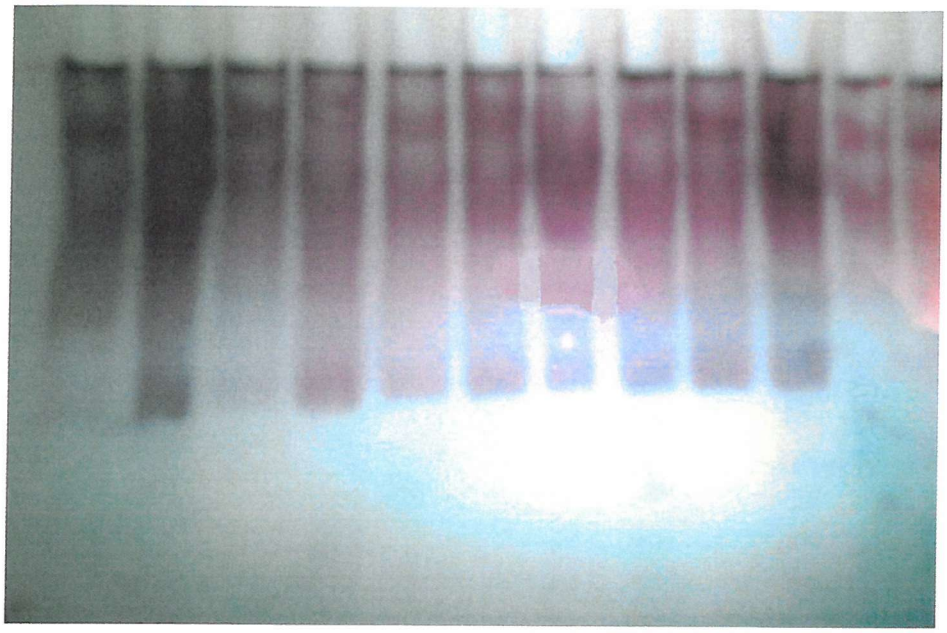

Plate 4.5 Variation in banding patterns of PRX

CHAPTER FIVE

Isolation and fusion of gametes and regeneration of plants

5.1 Introduction

Plant breeders are always looking for new techniques to use genetic variability to obtain desired type(s) of crops. This variability may be natural or produced by artificial methods but can be used in conventional and/or novel plant breeding methods.

Scientists have been trying to analyse the rice germplasm, including the wild relatives, because they are important reservoirs of genetic variability for biotic and abiotic stresses, increased biomass production and improved quality characteristics. However, several researchers have found out that hybrid sterility and breakdown are common in crosses within and between the cultivated species (*Oryza sativa* and *Oryza glabberrima*) of rice giving rise to genetic complications (Lynch *et al.*, 1991; Oka, 1991; IRRI, 1996). These genetic complications occur even in crosses between the same genepool with shared common forms. For example, crosses between *indica* and *japonica* always show reduced fertility and between *O. sativa* and *O. glaberrima* are at least partially sterile (Oka, 1991). These types of abnormalities occur not only in the crosses between *O. sativa* and *O. glaberrima*, but are also observed in other crosses, such as between *O. sativa* and *O. rufipogon* (IRRI, 1996). Many workers have tried to overcome difficulties in hybridization of crop plants by developing methods in addition to conventional methods and these include embryo rescue, protoplast fusion, direct gene transfer, plant transformation and *in vitro* fertilization.

Recently some researchers have given a new impetus in the method of *in vitro* fertilization and regeneration of plants from fused gametes with the aim of free gene transfer from one species to other. The technique has unique advantages over sexual hybridization, since it can be combined with unrelated genomes and can introduce

cytoplasmic male sterility into desired elite cultivars. Furthermore, the totipotency problems encountered at the time of somatic hybrids of Gramineae (Kisaka *et al.*, 1998) could be overcome efficiently using this technique, as they are not mesophyll protoplasts. Recently, the technique has successfully been utilised in several crop plants including maize, wheat, barley and *Nicotiana* where gametes were isolated and fertile plants were obtained from the fused gametes (Tian and Russell, 1997a; Dumas *et al.*, 1998; Kranz *et al.*, 1998; Kranz and Kumlehn, 1999). In many species isolation of gametes and regeneration of plants is now routine (Kranz *et al.*, 1998; Kranz and Kumlehn, 1999).

So far no report on the use of *in vitro* fertilization for rice improvement has been found. Therefore, because of the advantages associated with this technique, an attempt was made to develop methods to isolate gametes and to regenerate plants from the fused gametes initially from *indica* group of rice. The effects of different concentrations of calcium and various levels of pH in the fusion medium were investigated to achieve successful hybrids.

5.2 Materials and Methods

5.2.1 Plant materials

Rice seeds were sown in 15 cm plastic pots, containing John Innes Number 2 compost soil, after sterilisation in 15% domestos (commercial sodium hypo-chloride solution) for 15 minutes. Five accessions of *Indica* group of *Oryza sativa*, *viz.* Keora, Aug Meghi, Dhapa, Agali and Dud Kalam, identified as promising drought resistant types (Chapters 3 and 4), were used in this study. Plants were grown in a glasshouse under a light/dark cycle of 12/12±1 hours at day/night controlled temperatures of 28°/22°C to flower. Unpollinated spikelets from matured flowers were collected at the time of anthesis or just before the anthesis for isolation of sperm and egg cells. The maturity of the flower was visually measured when anthers were seen in yellow colour.

5.2.2 Isolation of egg cells

The isolation procedures of rice egg cells were as followed by Kranz *et al.* (1991a) and van der Maas *et al.* (1993). There are two steps involved in the isolation of female gametes. Firstly, the isolation of embryo sacs was carried out as a prerequisite of egg cells isolation. This was done by macerating ovules with cell wall degrading enzymes, such as cellulase and pectinase. Spikelets were surface sterilised in 95% alcohol for 1 minute, then in 15% bleach for 5 minutes and subsequently rinsed with sterilise distilled water three times. 50-70 ovules were dissected out carefully from the ovaries under a laminar flow cabinet and were incubated in 2 ml of different concentrations of maceration media contained $CaCl_2$, KH_2PO_4, $MgSO_4$, MES {2 – (N-morpholine) ethane sulphonic acid}, mannitol, pectinase, pectolyase Y 23 (Seishin), hemi-cellulase and cellulase (Sigma) at a pH of 5.5. The concentrations of different maceration mixture are given in Table 5.1 and Table 5.2. The ovules were incubated at 30°C with gentle shaking (30 rpm) in a water bath for 1, 2, 3, 4 and 5 hours (Table 5.1 and Table 5.2). The maceration mixture was passed through a Pasteur pipette to liberate embryo sacs after the appropriate incubation period.

Secondly, embryo sacs were collected from the first maceration mixture under a stereomicroscope (Nickon, Japan) with the help of a micropippette and transferred into a new enzymatic isolation mixtures containing the same concentration of enzymes as used in the maceration media (Table 5.1 and Table 5.2). Again, the materials were incubated at 30°C for 30 minutes with gentle shaking (30 rpm). The egg cells were then isolated from the loose embryo sacs with the help of a very thin glass needle and were analysed to determine the number of egg cells under a Phase Contrast microscope (Zeiss, Axioskop, Germany). The viability of isolated egg cells was estimated using fluorescein diacetate (FDA) following the procedure of Heslop-Harrison *et al.* (1984). The presence of cell wall was detected by staining with 1 mg/ml calcoflour white (CW), (Sigma) (Chen and chen, 1993). The comparative yield (%) of the isolated egg cells was determined by the following formula:

$$Yield\ of\ isolated\ egg\ cells\ (\%) = \frac{Number\ of\ egg\ cells\ isolated}{Number\ of\ ovules\ treated} \times 100$$

5.2.3 Isolation of sperm cells

The anthers, which were on the point of anthesis, were collected for isolation procedures. Pollen viability, as a standard for pollen quality, was tested using a fluorescein diacetate (FDA) reaction assay described by Heslop-Harrison *et al.* (1984). When ≥ 90% of the pollen was FDA positive, the pollen samples were used for the isolation procedure.

Initially, an artificial pollen germination study was carried out in order to isolate sperm cells for rice following the protocol of Tian and Russell (1997). Although a series of experiments was carried out following this method, the pollen grains failed to germinate and as a result isolation of sperm cells was not possible. Then the protocol of van der Maas *et al.* (1993a) and Theunis (1992) was followed. This was carried out in two steps. Firstly, pollen grains were collected from about 100 mg of anthers and were suspended in 2 ml of isolation medium at 30°C in a glass petri dish. The suspended pollen grains were broken by squashing them with a glass roller on a rough surface along with osmotic pressure, or shock, created by the isolation medium. Different concentrations of media compositions were used to improve the isolation procedure and are given in Table 5.4.

Secondly, the separation of sperm cells from the pollen grains was carried out by removing debris through filtration in a 25 μm nylon filter. The filtrate solution consisted of a mixture of cytoplasmic materials and the sperm cells. For further purification, the filtrated mixtures were centrifuged for 20 minutes at 13000 rpm, with an equal volume of preservation media {10 mM vitamin C (L-ascorbic acid) and 0.1 mM vitamin E (DL- α-tocopherol phosphoric acid, ester disodium salt), layered onto a density gradient of 10%, 30% and 70% (v/v) percoll. The sperm cells together with some cytoplasmic organelles were gathered in a narrow band on a layer between the original sample of squashed pollen grains and 10% percoll. All

procedures were carried out under a laminar flow cabinet and sperm cells were examined under the microscope as mentioned earlier. The viability of sperm cells was measured with 0.1% FDA (diluted in acetone at 1:300 in culture medium) and were observed under phase contrast microscope (Zeiss Axicope, Germany). The presence of cell wall was detected by Calcolflour White (Chen and chen, 1993).

The percentage of isolated sperm cells was calculated using the following formula:

$$\text{Percent of sperm cell} = \frac{\text{Number of sperm cells per microscopic field}}{\text{Number of pollen grains per microscopic feld} \times 2} \times 100$$

5.2.4 Fusion of sperm and egg cells

The fusion of isolated gametes of *Oryza sativa* species was carried out following the protocol of Kranz and Lorz, (1994) for maize. The fusion of egg and sperm cells was performed in two separate sets of experiments. In the first set of experiments, gamete fusion was attempted by varying calcium chloride concentration (4-15 mM $CaCl_2$) in the fusion media while keeping the pH constant (pH = 5.5). In the second set of experiments calcium chloride concentration was kept constant while pH of the media was varied from 5 to 11. The fusion media was composed of 4-15 mM $CaCl_2$, 3-5 mM KH_2PO_4, 3-5 mM MOPS {3-(N-Morpholino) propane sulphonic acid monosodium salt}, 0-5% mannitol with a pH of 5-11. The pH was adjusted with 1 M and 0.1M KOH.

For fusion, about 15 µl of fusion solution was placed on a cavity slide covered with mineral oil. One egg cell and several sperm cells were collected and transferred into the fusion medium using a micropipette. The cells were brought into contact manually using a thin glass rod and using a micromanipulator (Etabls. Beaudouin, Paris, France). The fusion of gametes was observed under an inverted microscope (Leitz-Diplan, Germany) attached to a computer with image-pro plus software. Each set of experiment was repeated for at least three times. The fusion products of isolated sperm and egg cells were sterilised with Millipore filter sterilisation

(Millipore Corporation ltd, Bedford, Ma., USA) and were transferred to a culture medium.

5.2.5 Plant regeneration from fused gametes

MS (Murashinge and Skoog, 1962) basal salt was used for plant regeneration of the fused gametes. The media was supplemented with 3% (w/v) sucrose, 1.5-2.5-mg/l 2,4-D, 50mg tryptophan 0.5mg/l nicotinic acid, and 0.5% (w/v) agarose. The pH of the media was adjusted to 5.7 before sterilisation. The media was transferred evenly (15 ml each) into 60-ml jars and was sterilised by autoclaving at 121°C and at a pressure of 1.1 kg per square centimetre. The fused gametes were picked up from the fusion medium with the help of a pasture pipette and transferred into jars under sterile conditions. The jar was incubated at 25±1° C in the dark for callus induction according to the procedure described by Lee *et al.* (1989) for protoplast culture of rice. The subculture was carried out once in every week in the dark for the production of enough calli and the data were recorded.

5.3 Results

5.3.1 Isolation of egg cells

The results of *in vitro* isolation of embryo sacs and egg cells are shown in Tables 5.1 and 5.2. After the incubation in enzyme maceration, the ovules were disintegrated into loose cells, and then embryo sacs were visible (Plate 5.1). Embryo sacs were released after 5 hours incubation in all media composition (Tables 5.1 and 5.2) and the egg cells in embryo sacs could clearly be distinguished from the bulk of other cells by their size and shape (Plate 5.2). After mechanical manipulation of isolated embryo sacs, egg cells were successfully isolated and 10 – 15 (20 – 30 %) egg cells were obtained from 50 ovules. The egg cells were always detected as single spherical shaped cells (Plate 5.3). The synergid cells were also spherical but smaller in size. The size of synergids was 30-35 μm, compared with egg cells of 45-50 μm. The highest number of egg cells (38.18 %) was obtained when ovules were incubated for 5 hours in a medium containing 2 % cellulase, 0.55 % pectinase, 5 mM $CaCl_2$, 5 mM KH_2PO_4, 0.7 mM $MgSO_4$, 3 mM MES {2-(N-morpholine) ethane sulphonic acid} and 10 % mannitol mixtures (Table 5.1). It was apparent from the results that the yield of egg cells was low. The viability of egg cells was detected by positive staining in a FDA test. The egg cells remained viable up to 24 hours after isolation. The egg cells were stained using CW, which showed that these were naked protoplasts.

Table 5.1 Effect of cellulase concentration on isolation of egg cell in rice

$CaCl_2$ mM	KH_2PO_4 mM	$MgSO_4$ mM	MES mM	Mannitol (%)	Cellulase (%)	Pectinase (%)	Number (%) of isolated egg cells*														
							1 hour			2 hours			3 hours			4 hours			5 hours		
							Ovules	Embryos	Egg cell	Ovules	Embryos	Egg cell	Ovules	Embryos	Egg cell	Ovules	Embryos	Egg cell	Ovules	Embryos	Egg cell
5	5	0.7	3	10	0.55	0.55	50	3 (6)	0 (0)	55	6 (10.91)	2 (3.4)	60	14 (23.33)	4 (6.67)	45	8 (17.78)	4 (8.89)	50	12 (24.0)	4 (8.0)
5	5	0.7	3	10	0.65	0.55	55	4 (7.27)	0 (0)	60	6 (10.00)	1 (1.67)	50	12 (24.00)	4 (8.0)	65	14 (21.54)	7 (10.77)	60	15 (25.0)	7 (11.67)
5	5	0.7	3	10	0.80	0.55	65	6 (9.23)	3 (4.6)	45	8 (17.78)	4 (8.89)	55	17 (30.91)	9 (16.36)	50	15 (30.0)	7 (14.0)	55	18 (32.73)	11 (20.0)
5	5	0.7	3	10	0.90	0.50	45	8 (17.78)	4 (8.89)	55	12 (21.82)	6 (10.91)	60	22 (36.67)	17 (28.33)	45	17 (37.78)	13 (28.89)	50	21 (42.0)	16 (32.00)
5	5	0.7	3	10	1.00	0.55	60	13 (21.67)	5 (8.33)	70	20 (28.57)	11 (15.71)	45	19 (42.22)	13 (28.89)	55	26 (47.27)	16 (29.09)	65	32 (49.23)	20 (30.77)
5	5	0.7	3	10	1.20	0.55	70	16 (22.86)	4 (5.71)	50	17 (34.0)	9 (18.0)	60	25 (41.67)	18 (30.00)	50	23 (46.00)	16 (32.00)	70	36 (51.43)	22 (31.43)
5	5	0.7	3	10	1.50	0.55	50	12 (24.0)	6 (12.0)	55	15 (27.27)	7 (12.73)	60	31 (51.67)	19 (31.67)	45	22 (48.89)	15 (33.33)	50	24 (48.0)	17 (34.0)
5	5	0.7	3	10	1.75	0.55	55	14 (25.45)	6 (10.91)	65	21 (32.31)	11 (16.92)	55	27 (49.09)	17 (30.91)	60	32 (53.33)	22 (36.67)	45	26 (57.78)	16 (35.55)
5	5	0.7	3	10	2.00	0.55	50	8 (16.0)	5 (10.0)	45	11 (24.24)	7 (15.55)	65	31 (47.69)	19 (29.23)	50	27 (54.0)	17 (34.0)	55	30 (54.54)	21 (38.18)

Constant pectolyase 0.2% (w/v), * Figure in parenthesis indicating percentage

Table 5.2 Effect of pectinase and other ingredient concentrations on isolation of egg cell in rice when 1% cellulase constant

$CaCl_2$ mM	KH_2PO_4 mM	$MgSO_4$ mM	MES mM	Mannitol (%)	Pectinase (%)	Number (%) of isolated egg cells*														
						1 hour			2 hours			3 hours			4 hours			5 hours		
						Ovules	Embryos	Egg cells	Ovules	Embryos	Egg Cells	Ovules	Embryos	Egg cells	Ovules	Embryos	Egg cells	Ovules	Embryos	Egg cells
5	5	0.7	3	10	0.55	60	13 (21.67)	5 (8.33)	70	20 (28.57)	11 (15.71)	45	19 (42.22)	11 (24.44)	55	24 (43.64)	12 (21.82)	65	32 (49.23)	18 (27.69)
6	5	0.7	3	10	0.65	65	15 (23.08)	7 (10.77)	55	22 (40.00)	10 (18.18)	55	29 (52.72)	17 (30.91)	50	26 (52.00)	16 (32.00)	45	24 (53.33)	15 (33.33)
7	5	0.7	3	10	0.75	70	19 (27.14)	9 (12.86)	55	23 (41.82)	12 (21.82)	50	27 (54.00)	16 (32.00)	55	28 (50.91)	18 (32.73)	60	35 (58.33)	21 (35.0)
8	5	0.7	3	10	0.85	45	11 (24.44)	6 (13.33)	50	21 (42.00)	11 (22.00)	65	34 (52.31)	21 (32.31)	45	24 (53.33)	15 (33.33)	55	31 (56.36)	19 (34.54)
9	5	0.7	3	10	1.0	55	14 (25.45)	6 (10.91)	40	15 (37.50)	9 (22.50)	50	25 (50.00)	15 (30.0)	60	33 (55.0)	21 (35.00)	45	25 (55.55)	15 (33.33)
10	5	0.7	4	9.5	0.75	40	13 (32.50)	5 (12.50)	65	29 (44.62)	15 (23.08)	55	29 (52.72)	18 (32.73)	45	23 (51.11)	15 (33.33)	50	26 (52.00)	15 (30.00)
7	5	0.7	4	9.5	0.75	60	14 (23.33)	6 (10.00)	50	23 (46.00)	9 (18.00)	45	22 (48.89)	13 (28.89)	50	26 (52.00)	15 (30.00)	65	35 (53.85)	21 (32.31)

Constant pectolyase 0.2% (w/v), * Figure in parenthesis indicating percentage

The effects of various concentrations of cellulase, pectinase, calcium chloride and incubation period on yield of egg cells are discussed below.

5.3.1.1 Effect of cellulase concentration on isolation of egg cells

The effects of cellulase concentrations on isolation of egg cells are shown in Table 5.1. It was apparent from the results that when the concentration of cellulase was increased, keeping pectinase and calcium chloride constant, there was increase in the release of embryo sacs and egg cells (Table 5.1) and thus indicated positive association of embryo sac/egg cells yields on cellulase concentrations (Fig. 5.1).

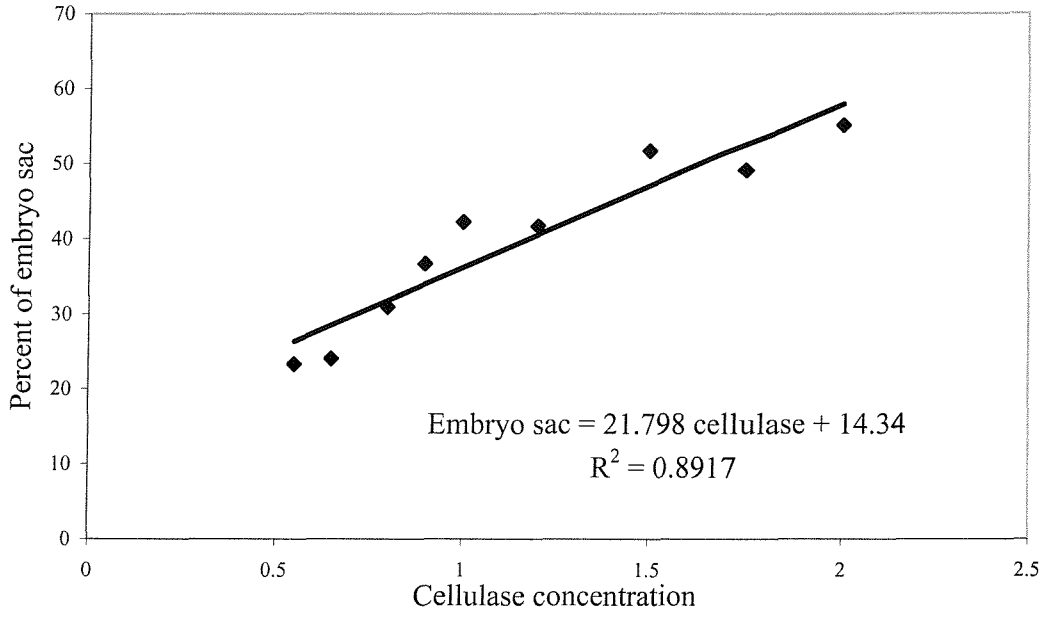

Fig. 5.1 Effect of cellulase concentration on embryo sac isolation of rice

5.3.1.2 Effect of pectinase and calcium concentration on egg cells isolation

The effects of pectinase and calcium concentrations on egg cells isolation are shown in Table 5.2 and Fig. 5.2. The results revealed that when the concentration of pectinase increased, keeping cellulase constant (1%), the number of released embryo sacs and egg cells increased (Table 5.2). Fig. 5.2 also confirmed the positive association of embryo sac/egg cells yields on pectinase concentrations. However, it was apparent from the table that higher concentration of calcium chloride had

reduced the yield of egg cells. Thirty five percent of egg cell was obtained when pectinase and calcium chloride were used at a concentration of 0.75% and 7 mM respectively at a constant (1%) concentration of cellulase (Table 5.2) and this can be considered as the best yield in this study. However, due to limitation of flowering, further studies could not be conducted with different concentrations of other ingredients.

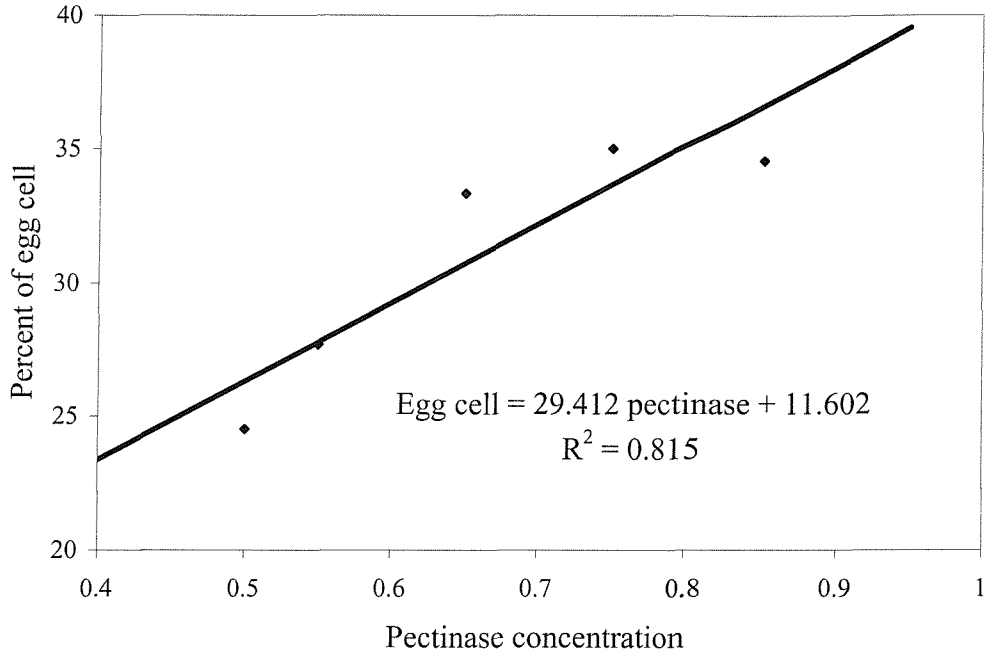

Fig. 5.2 Effect of pectinase concentration on egg cell isolation of rice

5.3.1.3 Effect of incubation period on egg cells isolation

It appeared from the results that in general, the efficiency of egg cell isolation was higher when the incubation period of ovules was longer (Tables 5.1 and 5.2). A considerable number of embryo sacs and egg cells were released after five hours of incubation in all media compositions used. The relationship between incubation time and percentage of isolated egg cells showed that egg cell yield increased with increased incubation time (hour) (Fig. 5.3).

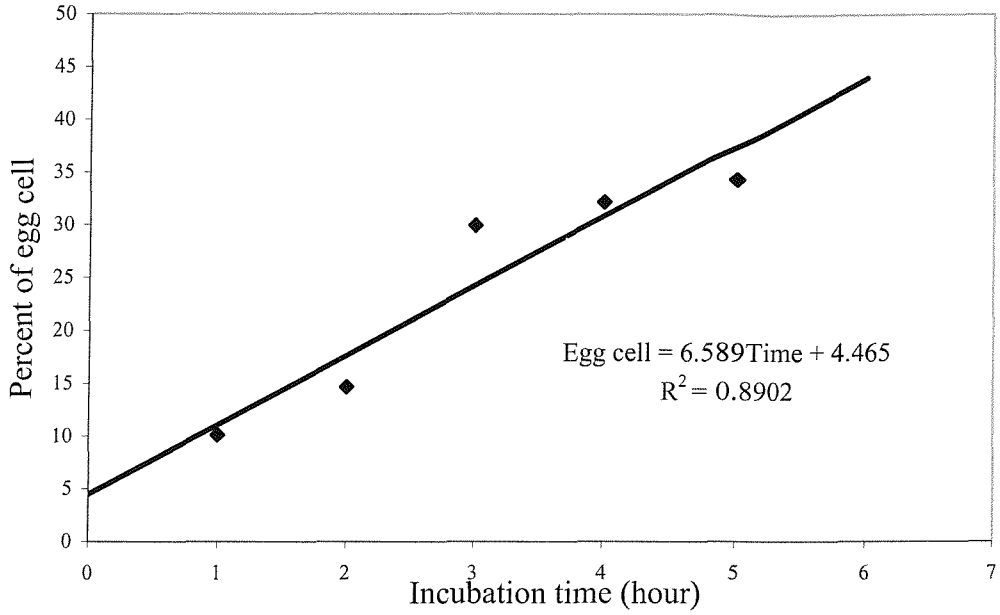

Fig. 5.3 Relationship between incubation time and percent of egg cell

5.3.2 Isolation of sperm cells

Artificial pollen germination experiments were carried out in order to isolate sperm cells of rice. The effect of different concentrations of boric acid, calcium chloride, calcium nitrate, potassium phosphate and sucrose from 0.0-2.0% (w/v) were tried with different lengths of time (6-24 hours) to find out the most effective concentration for pollen germination. Although up to 87% of pollen grains bursts were observed, not a single pollen grain was found to germinate (Appendix Tables 5.1 to 5.7). Hence experiments were carried out to determine the effects of various concentrations of boric acid, calcium chloride, calcium nitrate, potassium phosphate and sucrose on pollen burst. After 12 hours of incubation the results are described in the following sections.

5.3.2.1 Effect of boric acid concentration on pollen burst

The effects of different concentrations of boric acid on pollen burst are shown in Fig. 5.4. It was apparent from the results that the percent of pollen burst increased

with the increase of boric acid concentrations from 0.001% to 0.008% (w/v) and maximum pollen burst (81.69%) was observed at the concentration of 0.008% (w/v). However, a further increase in concentration of boric acid showed a decreasing tendency of pollen bursting which indicates that high concentration of boric acid had negative effect on rice pollen bursting.

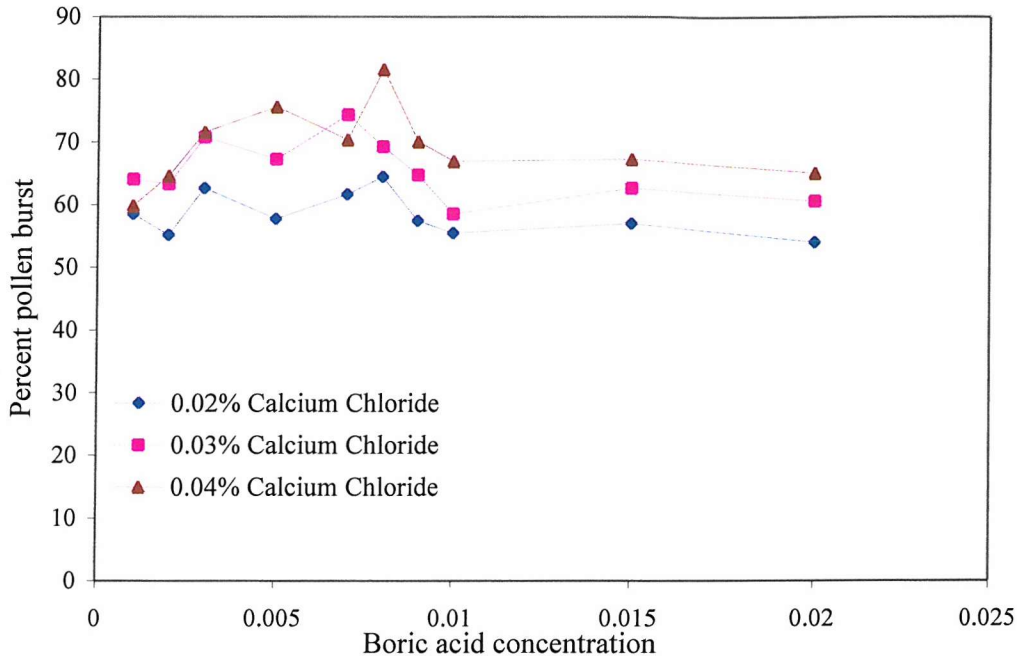

Fig. 5.4 Effect of boric acid and calcium chloride concentrations on rice pollen bursting

5.3.2.2 Effect of calcium chloride and calcium nitrate concentration on pollen germination

The percentage of pollen burst was higher (84.23%) after 24 hours of *in vitro* culture with calcium chloride at a concentration of 0.04% (w/v) (Fig.5.5 and Appendix: Tables 5.4 and 5.5). The results revealed that calcium concentration increased the pollen burst but better results were obtained when calcium chloride was used instead of calcium nitrate in all concentrations (Fig.5.5).

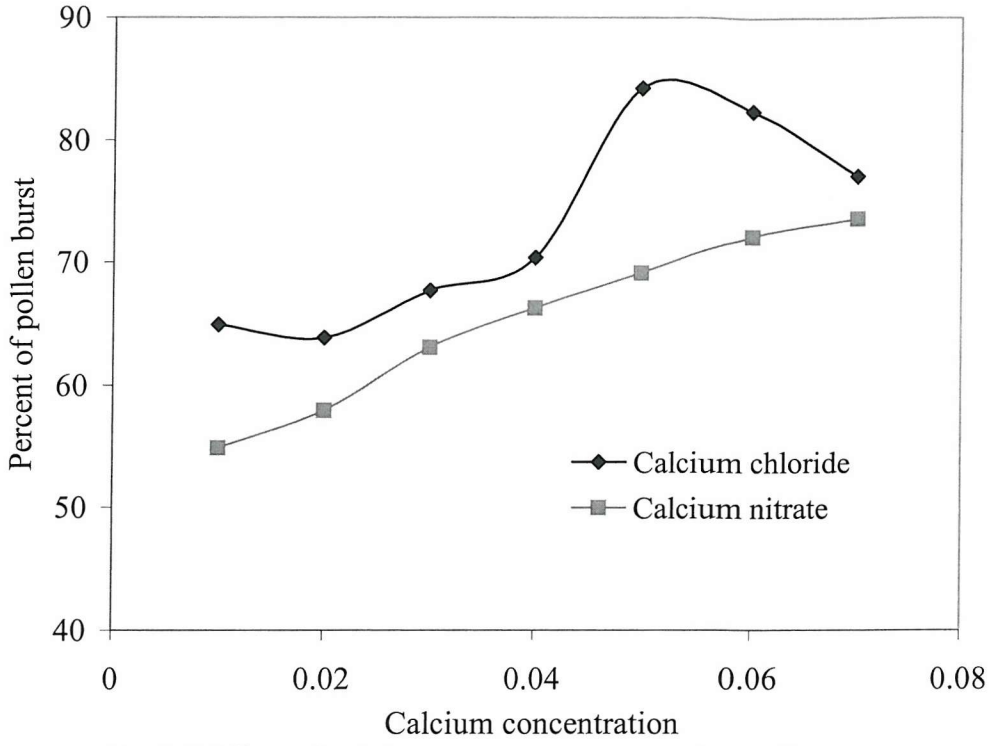

Fig. 5.5 Effect of calcium concentration on rice pollen bursting

5.3.2.3 Effect of potassium phosphate concentration on pollen germination

Like other ingredients, potassium had similar effect on pollen burst of rice (Table 5.3). It was observed that higher concentration of potassium in the culture media had negative effect on pollen burst (pollen burst decreased with the increase of concentration).

Table 5.3 Effect of potassium phosphate concentration on rice pollen burst

Concentration	0.0	0.01*	0.02	0.03	0.04	0.05	0.1	0.5
Number Pollen Grain	218.46 ±37.27	234.73 ±35.73	361.38 ±46.57	283.91 ±39.86	254.41 ±37.94	260.14 ±28.76	296.58 ±29.31	331.18 ±37.10
Pollen Burst	134.24 ±23.18 (61.47)	203.19 ±22.62 (86.75)	281.23 ±33.43 (77.84)	213.43 ±28.56 (75.26)	177.32 ±35.11 (69.68)	179.21 ±13.89 (68.85)	189.41 ±24.71 (63.85)	194.25 ±27.53 (58.61)
Germination	0	0	0	00	0	0	0	0

Figure in parenthesis indicate the percentage, 0.008% Boric acid, 0.04% Calcium chloride, 0.01% Magnesium sulphate and 15% Sucrose; * 25% Sucrose

5.3.2.4 Effect of sucrose concentration on pollen burst

Table 5.4 summarises the effects of different concentrations of sucrose in the isolation media for pollen bursting. The optimum concentration of sucrose in the media was found to be 15% (w/v) for pollen burst. At this concentration of sucrose, pollen burst was observed after 6 hours.

Table 5.4 Effect of sucrose concentration on percentage of rice pollen burst

Culture period/ concentration	Percent of pollen burst			
	6 hours	12 hours	18 hours	24 hours
10%	68.33	81.41	81.25	82.43
15%	73.05	84.23	79.89	84.79
20%	71.32	75.77	73.33	81.69
25%	76.68	78.29	77.46	86.42

Because of the problems experienced with the artificial germination of pollen, rice sperm cells were isolated by mechanical forces and the results of isolated sperm cells are presented in Table 5.5. The maximum number (17.8%) of sperm cells were obtained with a medium containing 0.008% (w/v) boric acid, 0.01% (w/v) potassium phosphate, 0.04% (w/v) calcium chloride and 20% (w/v) sucrose, 5 mM MOPS {3-(N-Morpholino) propane sulphonic acid monosodium salt} at a pH of 6.0. It was apparent from the Table 5.4 that the percent of sperm cells increased with the increase of boric acid and calcium in the isolation media. Better results even were obtained when calcium chloride was used instead of calcium nitrate as it was also observed in pollen burst. This implies that boric acid and calcium chloride concentrations have some effects on the isolation of sperm cells (Figs. 5.4 and 5.5).

Table 5.5 Effect of media composition and concentration on sperm cells isolation

Treat-ment	HBO_3 (%)	$Ca(NO_3)_2$ (%)	$CaCl_2$ (%)	KH_2PO_4 (%)	$MgSO_4$ (%)	MOPS (mM)	Sucrose (%)	Number of pollen grains	Sperm cells	% of sperm cells
1	0.01		0.03	0.01	0.02	5	10	357.1 ±19.6	50.12 ±12.5	7.02
2	0.01		0.04	0.02	0.01	7	15	296.6 ±25.7	86.97 ±16.3	14.7
3	0.01	0.05	-	0.02	0.01	7	20	387.7 ±36.2	115.8 ±21.8	14.9
4	0.008	-	0.04	0.01	0.01	7	20	407.4 ±29.4	144.7 ±18.7	17.8
5	0.005	0.05	-	0.01	0.01	7	25	396.9 ±18.4	124.1 ±23.8	15.6
6	0.008	-	0.05	0.02	0.01	7	10	434.5 ±13.8	153.6 ±26.7	17.7

After the isolation and centrifugation procedure, the sperm cells became spherical and appeared intact, with an average diameter of 7 μm (Plate 5.4). Testing with FDA revealed that more than 80% of the sperm cells showed a bright yellow/green fluorescent staining, indicating viability. The viability of sperm cells decreased with time and appeared to be about 50% after 5 hours' incubation at room temperature.

5.3.3 Fusion of sperm and egg cells

The results of *in vitro* fusion of gametes are shown in Table 5.6. The fusion process itself was very rapid, taking only 2-3 seconds, depending on the contact of egg and sperm cells. The best result in terms of fully fused gametes (55.56%) was obtained by a fusion medium containing calcium chloride concentration of 7mM with a pH of 7.5 (Table 5.6). The size of egg cell increased to a diameter of 60-70 μm and appeared spherical in shape (Plate 5.5) after fusion of sperm and egg cells.

Table 5.6 Chemical and pH effect on egg and sperm cells fusion of rice

$CaCl_2$ (mM)	KH_2PO_4 (mM)	MOPS (mM)	Man nitol (%)	pH	Number of egg cells contact with sperm cells	Not fused		Partially fused		Fully fused	
						Number	%	Number	%	Number	%
5	-	3	5	11	3.33±1.53	3.33±1.53	100.00	0	0	0	
5	-	3	1	11	4.33±1.53	4.33±1.53	100.00	0	0	0	0
5	5	3	1	11	5.13±1.42	5.13±1.42	100.00	0	0	0	0
10	5	3	1	11	5.00±1.00	5.00±1.00	100.00	0	0	0	0
15	5	3	1	11	4.00±1.00	4.00±1.00	100.00	0	0	0	0
5	5	3	1	10	3.67±0.57	3.67±0.57	100.00	0	0	0	0
6	5	3	1	10	4.33±1.15	4.33±1.15	100.00	0	0	0	0
7	5	3	1	10	4.67±0.57	4.67±0.57	100.00	0	0	0	0
5	5	3	1	9	4.33±1.53	4.00±0.33	92.31	0.33±0.57	7.69	0	0
6	5	3	1	9	4.67±1.53	4.33±1.15	92.86	0.33±0.57	7.14	0	0
7	5	3	1	9	4.00±1.00	3.67±0.58	91.67	0.33±0.58	8.33	0	0
8	5	3	1	9	5.00±1.00	4.33±0.57	86.67	0.67±0.57	15.38	0	0
5	5	3	1	8.5	5.67±1.52	5.00±0.67	88.23	0.67±0.57	11.76	0.33±0.57	5.88
6	5	3	1	8.5	5.33±1.15	3.67±0.67	68.75	1.00±0.00	18.75	0.67±0.57	12.5
7	5	3	1	8.5	5.00±1.00	3.33±0.57	66.67	1.00±0.00	20.00	0.67±0.57	13.33
6	5	3	1	8.0	5.33±1.52	2.67±0.57	50.00	1.33±0.57	25.00	1.33±0.57	25.00
7	5	3	1	8.0	3.67±0.57	1.00±0.0	27.27	0.67±0.57	18.18	1.67±0.57	45.46
8	5	3	1	8.0	4.67±2.08	2.33±1.53	50.00	1.00±0.0	21.43	1.33±0.58	28.57
4	5	3	1	7.5	6.00±1.00	2.67±0.57	44.44	1.33±0.57	22.22	2.00±1.00	33.37
5	5	3	1	7.5	4.67±0.57	1.67±0.57	35.71	1.33±0.57	28.57	1.67±0.57	35.71
6	5	3	1	7.5	6.33±1.52	2.00±0.00	31.58	1.33±0.57	21.05	3.00±1.0	47.37
7	5	3	1	7.5	3.00±1.00	0.67±0.57	22.22	0.67±0.58	22.22	1.67±0.57	55.56
8	5	3	1	7.5	5.00±1.00	1.33±0.58	26.67	1.67±0.58	33.33	2.00±1.00	40.00
9	5	3	1	7.5	5.67±0.57	2.33±0.57	41.18	1.67±0.58	29.41	2.00±1.00	35.29
10	5	3	1	7.5	5.33±0.57	2.00±0.0	37.5	1.33±0.57	25.0	2.00±1.00	37.5
5	5	3	1	7.0	6.33±1.53	2.33±0.58	36.84	1.67±0.57	26.32	2.33±0.57	36.84
6	5	3	1	7.0	6.00±1.00	2.00±0.00	33.33	1.33±0.57	22.22	2.67±0.57	44.44
7	5	3	1	7.0	5.33±0.57	2.00±0.0	37.5	1.00±0.0	18.75	2.37±0.57	43.75
6	5	3	1	6.5	6.33±1.53	2.33±0.57	36.84	1.67±0.57	26.31	2.33±0.58	36.82
7	5	3	1	6.5	5.00±1.00	2.00±1.00	40.00	1.00±1.00	20.00	2.00±1.00	40.00
6	5	3	1	6.0	4.67±0.57	1.67±0.57	35.71	1.67±0.57	21.43	2.00±0.0	42.86
7	5	3	1	6.0	4.67±0.57	1.67±0.57	35.71	1.33±0.57	28.57	1.67±0.57	35.71
6	5	3	1	5.5	6.33±1.53	2.33±0.58	36.84	1.67±0.58	26.32	2.33±1.15	36.84
7	5	3	1	5.5	4.00±1.00	1.67±0.57	41.67	1.00±00	25.00	1.33±0.58	33.33
6	5	3	1	5.0	5.00±0.50	3.00±0.57	60.00	1.00±0.00	20.00	1.00±0.00	20.00
7	5	3	1	5.0	5.33±1.53	2.67±0.67	50.00	1.33±0.58	25.00	1.33±0.58	25.00

It was observed from the study that the alignment of the egg and sperm cells and the concentration of medium for fusion were important. The egg cells started to swell at a calcium chloride concentration of 10 mM onward and it shrank when concentration of calcium chloride was 20 mM.

It appears from Table 5.6 that the number of fully fused gametes increased with the increase of pH of the medium. However, no fusion occurred when pH of the medium was more than 9 in different calcium concentrations, indicating the importance of pH in fusion.

5.3.4 Plant regeneration from fused gametes

Limited progress in regeneration of plants from fused gametes was made following the procedure of Lee *et al.* (1989) and Hodges *et al.* (1991). It was apparent from the results that fused gametes did not show any sign of callus or shoots even when different media were tried (Table 5.7). Unfortunately, most of the cultures were contaminated and as a result the experiments were abandoned (Table 5.7). As mentioned earlier, the limitation of flowering time did not allow investigation with varying concentrations of different ingredients. Therefore further investigation is needed to establish a suitable method for plant regeneration.

Table 5.7 Plant regeneration efficiency in different media

Concentration of 2,4-D	Number of fused product cultured	Contami-nated	Callus initiation	Unchanged fusion product	Percent regenerated
1.5 mg/l	3.83±1.17	2.17±0.98 (56.52%)	0	1.67±0.82 (43.48%)	0
2.0 mg/l	4.72±1.80	2.29±1.25 (48.48%)	0	2.57±0.98 (51.51%)	0
2.5 mg/l	4.86±1.68	2.71±1.25 (55.88%)	0	2.14±0.90 (44.12%)	0

5.4 Discussion

The recent success in *in vitro* fertilization and regeneration of plants from fused gametes have encouraged many researchers to employ these methods for crop improvement (Dumas *et al.*, 1998; Kranz and Kumlehn, 1999). This allured to the use of these methods in the present study, as there is no report available in this domain for rice. The objective of this study was to develop a suitable technique(s) for isolation of gametes and their fusion to facilitate gene transfer in rice where conventional breeding methods are unsuccessful. A method was developed to isolate viable sperm and egg cells of *Indica* type of *Oryza sativa* species and for *in vitro* gametic fusion using various concentrations of chemicals and levels of pH in the isolation and fusion media.

Like other angiosperms, the female gametophytes or embryo sacs of *Oryza* species contain gametes in a specialised structure, the ovule. Therefore, direct access to the female gametes of *Oryza* species is difficult and the egg cells are isolated either by micro-dissection or by enzymatic treatments of ovules, to ease the access to the female gametes. This was carried out by using the cell wall degrading enzyme mixtures, such as cellulase and pectinase for isolation of rice egg cells.

The effect of enzyme concentrations of the isolation media and the incubation period of the enzymatic maceration appears to be crucial in obtaining viable egg cells of rice. It was apparent from Figs. 5.1 and 5.2 that the yield of embryo sacs/egg cells increased with an increase of enzyme concentration such as cellulase and pectinase, which indicated the effect of enzyme concentrations on egg cell isolation. It was also apparent from the results that the isolation efficiency of embryo sacs and egg cells was low when the incubation period was low (Tables 5.1 and 5.2). It appears then that an incubation period of 3-4 hours was found to be appropriate for the isolation of embryo sacs of rice.

The enzymatic treatment of embryo sacs was critical because longer incubation periods caused other sporophytic cells to loose their cell walls to become protoplasts, which made egg cell identification difficult even though the yield of egg

cell isolation increased. The specific cellular organisation and the size of the cells could overcome the problem of the occurrence of sporophytic cells becoming protoplasts, as the sporophytic cells are specific. The size of the rice egg cell was about 45-50 μm (Plate 5.3) and the size of synergids about 30-35μm. The granular structure of the cytoplasm was probably due to starch grains and this has also been reported for the egg cells of maize (Kranz *et al.*, 1993; Rougier *et al.,* 1996; Kranz and Kumlehn, 1999), ryegrass (van der Mass *et al.*, 1993) and wheat (Yu and Jensen, 1985). The fluorescein diacetate staining showed that isolated egg cells were viable. However, it appears from the results that the efficiency of the egg cell isolation is low (Tables 5.1 and 5.2). This may be due to the damage of egg cells at the time of mechanical manipulation of embryo sacs.

Like other small grain cereals and grasses, rice pollen lose their viability soon after desiccation. For this reason, the pollen was used just before anthesis for the isolation of the sperm cells instead of the mature pollen after anthesis. It appears from the results that different concentrations of sucrose, boric acid and calcium chloride had effects on the isolation of rice sperm cells, because pollen burst was highly influenced by the concentration of different chemicals. For example, boric acid had a negative effect on pollen burst when a concentration of >0.01% (w/v) was used in the medium.

One difficulty in the isolation of sperm cells in squashing method was the separation of isolated sperm cells from debris. For this reason different separation procedures were conducted to purify the sperm cells. It appeared in the discontinuous percoll gradient that the unbroken and broken pollen grains were pelleted at the bottom of the tube where relatively high quantities of unbroken pollen were found. The sperm cells were collected from 10% layer of percoll. However, some cytoplasmic materials, which are believed to be of similar density, remained with sperm cells.

The present study showed that rice sperm cells were very small in size, on an average of 7 μm in diameter (Plate 5.4). The isolation of a large number of sperm cells have been reported from tri-cellular pollen species such as maize (Dupuis *et al.*, 1987), spinach (Theunis and van Went, 1989; Theunis, 1992) and perennial

ryegrass (van der Maas *et al.*, 1993a; van der Maas *et al.*, 1994). It appears from the above reports that all sperm cells have some common features; they are very small in size, from 1-10 μm in diameter, and after isolation, they become spherical and contain a very large nucleus and a small volume of cytoplasm. Present study also showed similar features of isolated sperm cells. Sperm cells do not have any cell wall, indicating that they are ready for fusion to their female counterparts (Dumas *et al.*, 1998).

Lifetime of sperm cells in natural conditions can be longer due to their biological functions. However, it appeared from the results that the viability of isolated sperm cells decreased with increased storage time especially when stored at room temperature. It revealed from FDA test that about 50% of viability was lost after 5 hours of storage. A longer viability of sperm cells was obtained when vitamin-E and vitamin C were added in the isolation media. The conditions for keeping sperm cells viable for a longer time are still to be improved. It appeared from the results that the yield of isolated sperm cells is low, as in egg cells, and needs to be improved.

A number of methods, such as fusion of isolated gametes mediated by calcium and pH level (Faure *et al.*, 1994; Kranz and Lorz, 1994; Tian and Russell, 1997), microinjecting isolated sperm cells into isolated embryo sacs (Matthys-Rochon *et al.*, 1994) and electro-fusion or chemically induced fusion (Kranz *et al.*, 1991a) have been applied for *in vitro* fertilization in angiosperms (Rougier *et al.*, 1996; Dumas *et al.*, 1998; Kranz and Kumlehn, 1999). In this study chemically induced fusion method was chosen. This was due to the fact that unlike electrofusion, fusing gametes under *in vitro* conditions using calcium and pH level in the fusion media, gamete specificity can be retained (Faure *et al.*, 1994).

Sperm cells were isolated without any cell wall degrading enzyme treatment and it is expected that sperm cells can easily be fused with somatic protoplasts and other gametoplasts because of their biological function. This has already been confirmed by results obtained with maize sperm cells (Kranz *et al.*, 1991a&b; Kranz and Lorz, 1994).

It comes out from the study that the fusion media was critical for efficient *in vitro* fertilisation or fusion. Fusion efficiency was low at lower concentrations of calcium chloride (about 4 mM, Table 5.6) and the optimum concentration of calcium chloride was found to be 7 mM in this study. Higher concentrations of calcium chloride (>10 mM) caused sperm and egg cells to shrink and this made the movement of gametes difficult. Results from this study showed that egg cells started to swell at a calcium chloride concentration of 10 mM and they completely shrunk at a concentration of 20 mM. This is in contrast to the results of Kranz and Lorz (1994), which reported that higher concentrations of calcium accelerated the fusion in maize. The pH level in the fusion media also influenced the fusion of gametes. The study showed frequent fusion at a pH of 7.5, which is almost neutral. At pH 8 and above the fusion rate decreased. No fusion occurred at a pH of 9 and above which again differed from the results of Kranz and Lorz (1994), as they found that higher pH promoted fusion efficiency. Thus high pH values had negative influence on fusion (Table 5.6) and pH values towards neutrality were favourable for the fusion of rice sperms and egg cells (Table 5.6).

Difficulties were encountered in the alignment of the sperm and egg cells, which could be overcome by electrofusion. Kranz and Lorz (1994) also reported this difficulty. The fusion of sperm and egg cells was a very rapid process, occurred within 2-3 seconds. This might be due to smaller size and turgor of the sperm cells. Kranz and Kumlehn (1999) also reported that the turgor of the cells and the differences in cell size are important factors for efficient cell fusion. However, the technique needs to be improved, especially the alignment of gametes, as yield was low.

A plant regeneration study from the fused products showed no sign of callus and shoots. Results indicate that the majority of the fused products were contaminated, which might be due to media sterilisation problems. Furthermore, fusion study was conducted under the microscope where the gametes were exposed to light, which may have reduced their viability. It was also reported by Dumas *et al.* (1998) that regeneration of fusion products was difficult to achieve and suggested that a more sophisticated protocol is required for the regeneration of this type of zygote.

In conclusion, methods for isolation of gametes and their *in vitro* fusion were developed although the success rate was very low. These methods could be useful for free gene transfer between and within *Oryza* species and in sub-species. The recent progress in *in vitro* fertilization of isolated sperm, egg and central cells of maize (Kranz *et al.*, 1998) and other species, has shown the possible mechanisms of adhesion, recognition, fusion and early embryo development from fused gametes. This would provide adequate opportunities to manipulate isolated and fused gametes for transfer of desirable traits from *Oryza* species and in sub-species, and also in other species. In addition, the techniques open up new ways for chromosome transfer between two gametes in plants and create new opportunities to study the interaction between nucleus and cytoplasmic organelles (Dumas *et al.,* 1998). Although *in vitro* gametic fusion of rice was achieved using different concentrations of calcium chloride at various pH levels in the fusion media, the regeneration of plants from fused gametes was not achieved and still need further work.

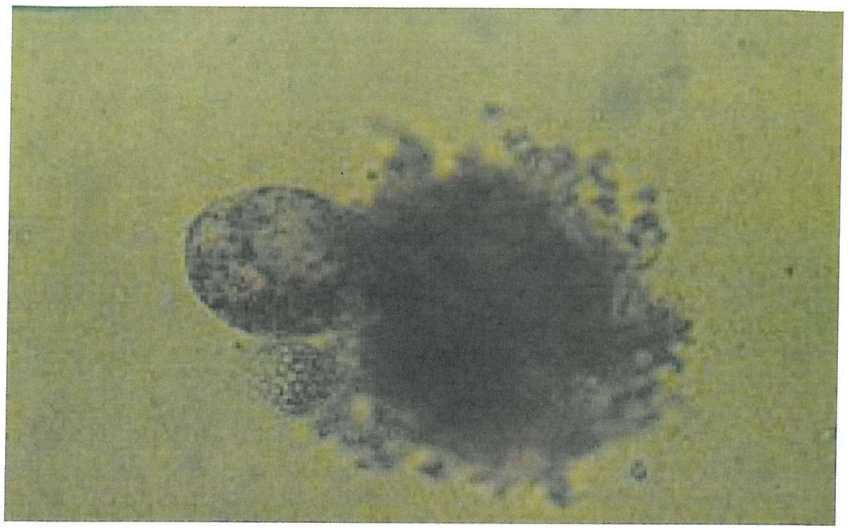

Plate 5.1 Isolated embryo sac X 300

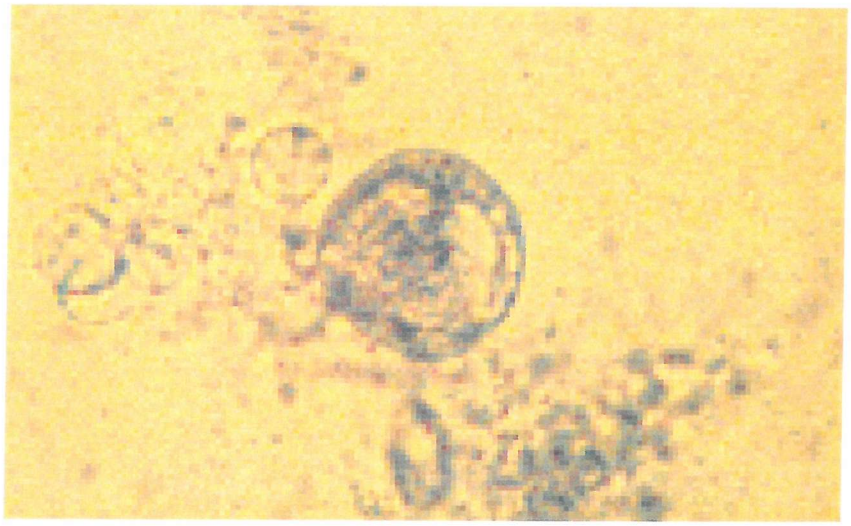

Plate 5.2 Embryo sac showing egg cell, central cell and synergids X 475

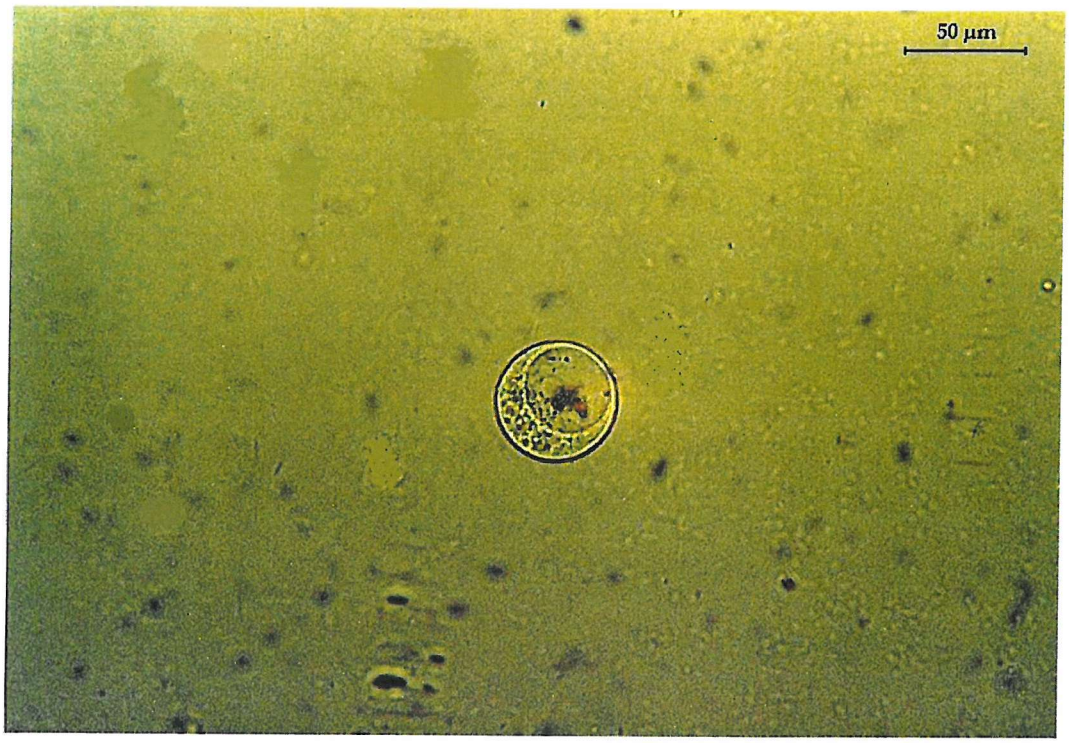

Plate 5.3 Isolated egg cell of rice X 300, bar= 50 µm

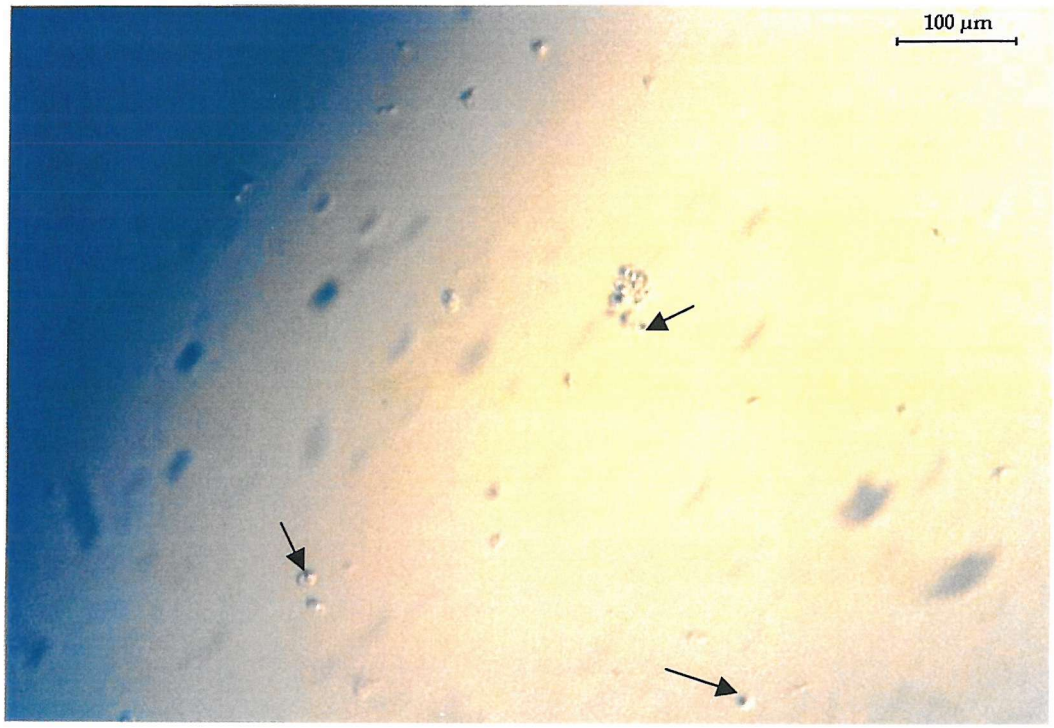

Plate 5.4 Isolated sperm of rice X 100, bar= 100 µm, Arrow showing sperm cell

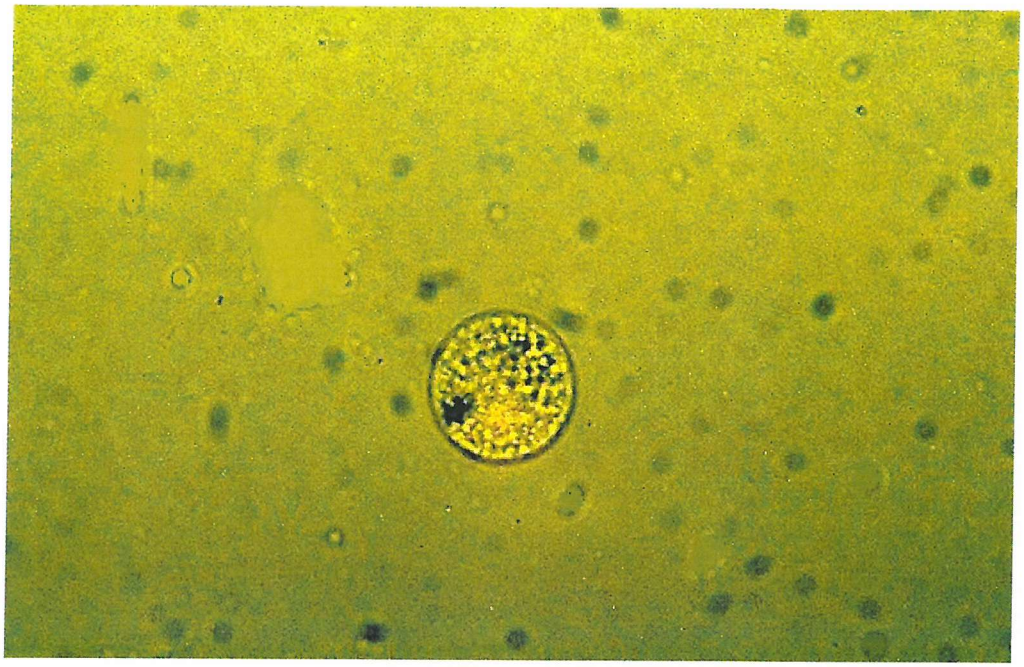

Plate 5.5 Fused egg and sperm cells X 325

CHAPTER SIX

6.1 General discussion and conclusions

The genetic variability, whether it is natural or artificial, is the basic requirement for modifications of crop plants to improve their suitability for cultivation. Plant breeders usually examine the existing available germplasm for variability and select the desirable type(s) for use in crop improvement programmes. Over the years, plant breeders have become more competent in their approach to developing new varieties of crops including rice, through the use of conventional and novel techniques.

Rice has enormous potential for production in tropical and sub-tropical areas but it has several constraints. One of the major constraints is its susceptibility to drought, which causes about 22.9% loss of productivity (Herdt, 1991). Nevertheless, several workers have already reported variability in the response to abiotic stresses within and between *Oryza sativa* and its sub-groups (Vaughan, 1994; IRRI, 1995). The use of this variability for drought resistance could immensely be valuable for rice improvement programmes.

In the present study, germplasm obtained from different ecosystems of Bangladesh were evaluated to identify potential drought resistant types through physio-morphological and isozyme studies with the aim of transferring genes to the high yielding and good quality rice cultivars. Initially, pot-screening experiments were carried out to evaluate physio-morphological characters. In this study visual drought score, days required to seedling emergence, tiller/plant, shoot length, root length, root-shoot length ratio, shoot dry weight (g) per plant, root dry (g) per plant, root-shoot dry weight ratio, total dry weight per plant, leaf area, leaf length, leaf width, stomata number, stomata conductance, ET and water use efficiency were used as parameters for physio-morphological characterisation.

The study involving pot-plant experiments revealed a significant variability within and between the accessions investigated. The supporting evidences came from the

results of both physio-morphological and biochemical experiments. It is apparent from the physio-morphological studies that the parameters, such as visual drought score, stomata behaviour, leaf rolling, root-shoot dry weight ratio, water use efficiency and evapotranspiration were important characters to estimate these variabilities.

In visual score leaf rolling of the accessions studied were seen to show different responses to water deficit. Six accessions from experiment-1 and 12 accessions from experiment-2 were found to have lower score. These might be potential genotypes for drought resistance types. It has also been noted that the leaf rolling was also seen to play role in light interception and transpiration during water stress.

The stomata also played an important role for water uptake as their conductance was reduced to prevent excessive water loss during water stress conditions. Turner and Burch (1983) and Azam-Ali and Squire (2002) also reported that crop species, including rice, close their stomata when water deficits occur in order to maintain high plant water status by minimising transpiration.

Furthermore, plants can maintain high water status by absorbing more ground water through the larger root systems and usually this can be ascribed to greater root-to-shoot dry weight ratio. The study also showed that higher root-shoot dry weight and root-shoot length ratios were inversely correlated with visual drought score *i.e.* leaf rolling. This indicated that the accessions (7 and 10 accessions in experiment-1 and experiment-2 respectively) had larger root systems and could uptake more ground water and maintain high water balance in the plant for their growth and development.

It is apparent from the results of water use efficiency that dry matter production was higher in some accessions for their internal mechanism which was influenced by leaf, shoot and root activities.

The overall results of the two experiments showed similar in most parameters studied and any difference observed in the parameters between the two pot-plant experiments might be due to environmental differences and because of longer duration of experiment-2. The uni- and multivariate statistical analyses and cluster analysis for pot experiments of these physio-morphological parameters in both studies showed consistently that four accessions Dud Kalam, Dhapa, Hogla Pata, and Keora might have the drought resistance characters. However, it is evident from the above study that these selections were subjected to both environmental and genetical effects.

The intention of this investigation was to identify genotypes for drought resistance types for crop improvement. The present study on isozyme investigation using five enzyme systems namely: ACP, GOT, EST, PRX and PGI clearly indicated that isozyme systems, especially EST, PRX and PGI were highly polymorphic and showed variability at molecular levels (Table 4.2). The results established that 51 accessions studied were certainly categorised into distinct isozyme phenotypes with different enzyme systems according to their genetic similarity.

This was supported from both statistical and cluster analyses and only three accessions, namely: Dhapa, Dud Kalam and Dud Mona were consistently grouped together in different isozymes (Table 4.3) as shown in the dendrogram (Fig. 4.7). These three accessions therefore could be distinct genotypes, which could be treated potential drought resistant types.

Comparison of cluster groups between the physio-morphological and isozyme studies revealed that majority of the accessions did not correspond to their respective groups due to the differences between the expressed phenotype(s) and actual genotype(s). This implied that cluster groups might be composed of different genotypes with similar phenotypic expressions and/or isozyme markers showing higher genetic diversity than the physio-morphological characteristics. Another reason may be the different number of accessions used in the two experiments. However, several authors have reported variations between morphological,

biochemical and molecular markers in rice (Suh and Morishima, 1997; Thanh *et al.*, 1999). Corresponding studies of isozyme and physio-morphological characteristics showed that only two promising drought resistant accessions, *viz*: Dhapa and Dud Kalam were consistently linked with the results of all the studies carried out (Table 4.6) and these two accessions constantly showed drought resistant characteristics. These two accessions expressed genetic similarity according to isozyme and in a conservative way could be considered as most promising drought resistant types. Therefore, it can be concluded that these two accessions might be identified as drought resistant types and could be utilised in a breeding programme to develop drought resistant types.

As mentioned earlier that the ultimate objective of this study was to transfer drought resistance gene(s) to high yielding cultivar(s), once the drought resistance genotypes were identified. Although several conventional and novel methods have been used to transfer desirable traits from related species to cultivated varieties, no record has been found for gametic fusion through *in vitro* methods in rice. However, a few researchers have succeeded in isolating and fusing gametes and regenerated plants in maize. This method has some advantages over the protoplast fusion and other techniques such as embryo rescue. So it seemed appropriate to use it to facilitate gene transfer between *Oryza sativa* species and sub-species through *in vitro* fertilization of isolated gametes and plant regeneration from the fused gametes. However, as a pre-requisite of *in vitro* fertilisation, methods for the *in vitro* isolation of viable male and female gametes of *Oryza sativa* species were developed.

The effects of different chemical compositions and concentrations were observed during the isolation of sperm and egg cells and fusion of gametes of *Oryza sativa* species. It was clear from this study that enzyme concentrations, especially cellulase and pectinase, had positive effects on rice egg cell isolation. The best yield of egg cells was achieved when calcium chloride, cellulase and pectinase were used at concentrations of 7 mM, 1 % (w/v) and 0.75 % (w/v), respectively. The incubation period also had an influence on the yield of embryo sacs/egg cells because even at

the same concentration of enzyme mixtures, the yield of embryo sacs/egg cells increased with a longer incubation period.

It seemed that the different compositions of media, and concentrations of calcium, boric acid and potassium, played a vital role in pollen bursts and sperm cell isolation. Boric acid concentration had some effects on sperm cell isolation when concentration increased beyond 0.01% in the isolation media. Similarly the rate of sperm cell isolation was higher when sucrose concentration was increased. However, these results were not comprehensive, as the study was conducted not beyond 25% of sucrose concentration.

The results obtained from *in vitro* fusion of sperm and egg cells showed that appropriate media compositions and pH levels were very important for the fusion of gametes. The most frequent fusion of gametes occurred when calcium chloride was used at a concentration of 7 mM with a pH of 7.5 in the fusion medium. When the concentration of calcium was increased to more than 10 mM in the fusion media, the movement of gametoplasts became difficult and egg cells started to swell and as a result fusion frequency decreased. Fusion frequency also decreased at lower concentrations (4-5 mM) of calcium chloride. It was shown from this study that pH level also influenced the fusion of sperm and egg cells. In this study frequent fusion of gametes did not occur at lower, or higher pH, but frequent fusion occurred towards neutral pH.

It is obvious from the study that *in vitro* fertilization of isolated sperm and egg cells of *Oryza* can be carried out by the manipulation of concentrations of calcium chloride and pH levels in the media. Therefore, it can be concluded from these studies that isolation of sperm and egg cells and fusion of these gametes of rice were achieved even though the success rate was found to be low in the present study.

In this study, plant regeneration from the fused gametes were attempted but could not be achieved because of the limitation of time and materials. No signs of callus or shoots were noted but since the experiments were carried out for a brief period due

to shortage of time. Therefore, further improvement of the method is needed to determine the suitable media for the regeneration of plants from fused gametes.

In summary, a few accessions of rice were identified for drought resistance from the physio-morphological study. The analysis showed that the variability observed in the physio-morphological studies were not entirely due to environmental effects alone but some were distinctly different genetically, which were confirmed by isozyme study. However, it was apparent from both studies that at least two genotypes (Dhapa and Dud Kalam) have shown drought resistance characters. These could be used for gene transfer to develop drought resistant type(s) of rice. Isolation of sperm and egg cells studies showed that they were affected by different compositions and concentrations of chemicals. These effects have also been noted in the fusion process of gametes. The protocols for the isolation of gametes and fusion for *Oryza* species could be useful in opening up new ways to overcome the barriers which occur during crosses between and within *Oryza* species and subspecies. However, in this study methods developed for both *in vitro* isolation of gametes and their *in vitro* fusion gave lower rate of success. The protocols developed in this study need to be improved for routine isolation of gametes and their *in vitro* fusion. Although the regeneration of plants from *in vitro* fused products was attempted no success was achieved and more work is needed to devise a simple protocol for regeneration of *in vitro* fused gametes.

References

Abdullah, R.; E.C. Cocking, and J.A. Thompson. 1986. Efficient plant regeneration from rice protoplasts through somatic embryogenesis. *Bio/Technol.* 4:1087-1090

Ackerson, RC; D.R. Krieg, and F.J.M. Sung. 1980. Leaf conductance and osmoregulation of field grown sorghum. *Crop Sci.* 20:10-14.

Alikani, M.; J. Cohen, and G.D. Palermo. 1995. Enhancement of fertilization by micro-manipulation. *Curr. Opin. Obs. Gyn.* 7: 182-187.

Anand, L. 1998. Assessing diversity using molecular and other techniques in tropical fruit crops. *In*: Arora, R.K. and V. Ramanatha Rao (eds). Tropical fruits in Asia: Diversity, Maintenance, Conservation and Use. International Plant Genetic Resources Institute. Pp 79-89.

Aradhya, M.K.; L.K. Yee; F.T. Zee, and R.M. Manshardt. 1998. Genetic variability in *Macadamia. Genetic Resources and Crop Evolution*. 45: 19-32.

Armenta-Soto, J.; T.T. Chang; G.C. Loresto, and J.C. O'Toole. 1983. Genetic analysis of root characters in rice (*Oryza sativa* L.). *Soc. Adv. Breed. Res. Asia Oceania J.* 15:103-116.

Azam-Ali, S.N., and G.R. Squire. 2002. Principles of tropical agronomy. CAB International, UK and USA.

Azam-Ali, S.N.; J. Aguilar-Manjarrez, and M. Bannayan-Avval. 2001. A global mapping system for Bambara Groundnut production. FAO Agricultural information management series. No. 1. FAO, Rome.

Baily, D.C., 1983. Isozymic variation and plant breeders' right's. Pp. 425-441. *In*: S.D. Tanksley and T.J. Orton (eds). Isozymes in plant genetics and breeding. Part A, Elsevier, New york.

Bashar, M.K.; R.K. Das; M.A. Islam, and N.M. Main. 1990. Genetic variability in midday leaf water potential (LWP) of irrigated rice. *International Rice Research Newsletter.* 15(5): 12

Bennett, J. 2001. Summing up: cutting edge science for rice improvement-breakthroughs and beneficiaries. *In*: Rice Biotechnology: Improving yield, stress tolerance and grain quality (eds.),A. Goode and D. Chadwick. Novartic Foundation. John Wiley & Sons Ltd. New York, Chichester, Brisbane, Singapore, Toronto pp. 242-251.

Bidinger, F.R.; V. Mahalakshmi; B.S. Talukdar, and G. Alagarswamy. 1992. Improvement of drought resistance in pearl millet. *In*: Drought resistance in crops with emphasis on rice. IRRI, Los Banos, Philippines. pp. 357-375.

Blum, A. 1974. Genotypic responses in sorghum to drought stress. II. Leaf tissue water relations. *Crop Sci.* 14:691-693.

Blum, A. 1980. Drought avoidance in wheat and its rapid estimation by remote infra-red thermal leaf canopy measurements. Proc. 3rd Int. Wheat Conf., May 22-June, Madrid, Spain.

Blum, A. 1983. Genetic and physiological relationship in plant breeding for drought resistance. *Agrc. Water Magmt.* 7: 195-205.

Blum, A. 1988. Plant Breeding for Stress Environments. CRC Press, Boca Raton FL.

Blum, A. 1989. Osmotic adjustment and growth of barley genotypes under drought stress. *Crop Science.* 29: 230-233.

Boyer, J.S. 1980. Water and the genetic improvement of soybean yields in the Midweat. *In*: P.J. Kramer and C.D. Raper, Jr., eds. Crop reactions to water and temperature stress in humid, temperate climates.

Boyer, J.S. 1982. Plant productivity and environment. *Science.* 218: 443-448.

Brar, D.C., and G.S. Khush. 1986. Wide hybridization and chromosome manipulation in cereals. *In*: D.A. Evans, W.R. Sharp and P.V. Ammirat (eds). Handbook of plant cell culture. Macmillan. NY. 4:221-263

BRRI. 1997. Bangladesh Rice Research Institute: serving the nation. BRRI Publication No. 119

Busscher, W.J.; R.E. Sojka, and C.W. Doty. 1986. Residual effects of tillage on coastal plain soil strength. *Soil Sci.* 141:144-148.

Chaboud, A., and R. Perez. 1992. Generative cells and male gamtes: isolation, physiology and biochemistry. *International review of cytology.* 140: 205-232.

Chamberlain, J.R. 1998. Isozyme variation in *Calliandar calothyrsus* (Leguminosae): its implications for species delimitation and conservation. *American J. Botany.*85(1):37-47.

Champoux, M.C.; G. Wang; S. Sarkarung; D.J. Mackill; J.C. O'Toole; N. Huang, and S.R. McCouch. 1995. Locating genes associated with root morphology and drought avoidance in rice via linkage to molecular markers. *Theor. Appl. Genet.* 90:969-981.

Chandra Babu, R.; H.G. Zheng; M.S. Pathan; M.L. Ali, and A. Blum. 1996. Molecular mapping of drought resistance traits in rice. pp.637-642. International Rice Research Institute. 1996. Rice genetics III. Proceedings of the Third International Rice Genetics Symposium, 16-20 Oct. 1995. Manila (Philippines): IRRI.

Chandraratna, M.F. 1964. Genetics and Breeding in Rice, Longmans, Green and Co ltd. London.

Chang, T.T.; G.C. Loresto, and O. Tagumpay. 1972. Agronomic and growth characteristics of upland and lowland varieties. pp. 648-661. *In*: Rice Breeding. IRRI, Los Banos, Philippines.

Chang, T.T.; G.C. Loresto, and O. Tagumpay. 1974. Screening rice germplasm for drought resistance. *Soc. Adv. Breed. Res. Asia Oceania J.*, 6(1):9-16.

Chang, T.T., 1976. The origin, evolution, cultivation, dissemination and diversification of Asian and African rice. *Euphytica* 25:425-441.

Chang, T.T.; G.C. Loresto; J.C. O'Toole, and J.L. Armenta-soto. 1982. Strategy and methodology of breeding in rice for drought prone areas. 217-244. *In*: Drought resistance in crops with emphasis on rice. International Rice Research Institute, Los Banos, Manila, Philippines

Chang, T.T.; J.L. Armenta-Soto; C.X. Mao; R. Peris, and G. C. Loresto. 1987. Genetic studies on the components of drought resistance in rice (*Oryza sativa* L.). *In: Rice Genetics*. pp.387-398. IRRI, Los Banos, Philippines.

Chaubal, R., and B.J. Reger. 1990. Relatively high calcium is localised in synergid cells of wheat ovaries. *Sex. Plant Reprod.* 3:98-102.

Chaubal, R., and B.J. Reger. 1993. Pre-pollination degeneration in mature synergids of pearl millet: an examination using antimonate fixation to localize calcium. *Sex. Plant Reprod.* 7:225-238.

Chen, Y.C., and C.S. Chen. 1993. Use of fluorescent staining to monitor the temporal pattern of cell resynthesis in *Ulva fasciata* (Chlorophyta: Ulvales, Ulvaceae) protoplasts. *Japan J. Phycol.* 41: 237-242.

Clifford, S.C; I.M. Stronach; C.R. Black; P.R. Singleton-Jones; S.N Azam-Ali, and N.M.J Crout. 2000. Effects of CO_2, drought and temperature on the water relations and gas exchange of groundnut (*Arachis hypogaea*) stands grown in controlled environment glasshouses. *Physiologia plantarum*. 110:78-88

Clover, G.R.G.; K.W. Jaggard; H.G. Smith, and S.N. Azam-Ali. 2001. The use of radiation interception and transpiration to predict the yield of healthy, droughted and virus-infected sugar beet. *J. Agric. Sci.* 136:169-178

Cocking, E.C. 1960. A method for isolation of plant protoplasts and vacuoles. *Nature* 187: 962-963

Collinson, S.T.; E.J. Clawson; S.N. Azam-Ali, and C.R. Black. 1997. Effects of soil moisture deficits on the water relations of bambara groundnut (*Vigna subterranea* L. Verdc.). *J. Exptal. Botany*. 48(309):877-884

Cruz, R.T; J.C. O'Toole; M. Dingkuhn; E.B. Yambao; M. Thangaraj, and S.K. De Datta. 1986. Shoot and root responses to water deficits in rainfed lowland rice. *Aust. J. Plant Physiol.* 13: 567-575.

Datta, S.K. 1981. Principles and practices of rice production. John Willy & sons, New York, Toronto, Singapore

Datta, S.K.; K. Datta, and I. Potrykus. 1990. Fertile indica plants from protoplasts isolated from microspore derived cell suspensions. *Plant cell rep.* 9: 253-256.

David, C.C. 1991. The world Rice economy: Challenges Ahead. pp1-18. *In: Rice Biotechnology*. (Ed). Khush, G.S. and G.H. Toenniessen. CAB International and International Rice Research Institute.

Dumas, C., and H.L. Mogensen. 1993. Gametes and fertilization: maize as a model system for experimental embryogenesis in flowering plants. *Plant Cell*, 5:1337-1348.

Dumas, C.; F. Berger; J.E. Faure, and E. Matthys-Rochon. 1998. Gametes, Fertilization and Early Embryogenesis in flowering plants. *Advances in Botanical Research*. 28:231-261.

Dupuis, I.; P. Roeckel; E. Matthys-Rochon, and C. Dupas. 1987. Procedure to isolate viable sperm cells from corn (*Zea mays* L.) pollen grains. *Plant Physiology*. 85:876-878

Ekanayake, I.J.; J.C. O'Toole; D.P. Garrity, and T.M. Masajo. 1985. Inheritance of root characters and their relations to drought resistance in rice. *Crop Sci.* 25:927-933.

Ekanayake, I.J.;D.P. Garrity; T.M. Masajo, and J.C. O'Toole. 1985a. Root pulling resistance in rice: inheritance and association with drought tolerance. *Euphytica*.34:905-913.

El-Husseini, S. N. 1993. A model for irrigation scheduling with saline water. PhD Thesis. University of Southampton, UK.

FAO. 1979. Yield response to water. FAO irrigation and drainage papers number 33.

FAO. 2001. World cereal production. Abstracted from FAO web page 2001.

Faure, J.E.; H.L. Mogensen; C. Dumas; H. Lorz, and E. Kranz, 1993. Karyogamy after electrofusion of single egg and sperm cell protoplasts from maize: Cytological evidence and time course. *The Plant Cell*. 5:747-755.

Faure, J.E.; C. Digonnet, and C. Dumas. 1994. An *in vitro* system for adhesion and fusion of maize gametes. *Science* 263:1598-1600.

Fischer, R.A., and M. Sanchez. 1979. Drought resistance in spring wheat cultivars. II. Effects on plant water relations. *Aust. J. Agric. Res.* 29:897-912.

Flowers, T.J.; M.L. Koyama; S.A. Flowers; Chinta Sudhakar; K.P. Singh, and A.R. Yeo. 2000. QTL: their place in engineering tolerance of rice to salinity. *J. Exp. Botany*, Vol. 51(342): 99-106.

Fujimura, T.; M. Sakura; H. Akagi; T. Negishi, and A. Hirosa. 1985. Regeneration of rice plants from protoplasts. *Japan. J. Breed.* 35: 48-49.

Fukai, S., and M. Cooper. 1995. Development of drought resistant cultivars using physio-morphological traits in rice. *Field Crops Research.* 40: 67-86.

Garrity, D.P.; C.Y. Sullivan, and W.M. Ross. 1982. Alternative approaches to improving grain sorghum productivity under drought stress. *In*: Drought resistance in crops with emphasis on rice. IRRI, Los Banos, Manilla, Philippines. pp.339-356.

Garrity, D. P., and O'Toole, J. C. 1994. Screening rice for drought resistance at the reproductive phase. *Field crops research.* 39(2-3): 99-110.

Garrity, D. P., and O'Toole, J. C. 1995. Selection for reproductive stage drought avoidance in rice, using infrared thermometry. *Agronomy Journal.* 87(4): 773-779.

Gauthier, P.; R. Lumaret, and B. Alain. 1998. Genetic variation and gene flow in Alpine diploid and tetraploid populations of *Lotus* (*L. alpinus/ L. corniculatus*). I. Insights from morphological and allozyme markers. *Heredity.* 80: 683-693

Glaszmann, J.C. 1985. A varietal classification of Asian cultivated rice (*Oryza sativa* L.) based on isozyme polymorphism. *In*: Rice Genetics II. IRRI, Los Banos, Manila, Philippines. pp.83-90.

Glaszmann, J.C. 1987. Isozymes and classification of Asian rice varieties. *Theor. Appl. Genet.* 74:21-30.

Gomathinayam, P.; K. T. Ingram, and M. A. Maguling. 1988. Pot screening for drought tolerance in rice. *International Rice Research Newsletter.* 13(6): 19

Grist, D.H. 1953. Rice. Longmans, Green and Co. London, New York and Toronto.

Grumet, R.; R.S. Albrechtensen, and A.D. Hanson. 1987. Growth and yield of barley isopopulations differing in solute potential. *Crop sci.* 27:991-995.

Guiderdoni, E., and H. Chair. 1992. Plant regeneration from haploid cell suspension-derived protoplast of Mediterranean rice (*Oryza sativa* L. cv Miara). *Plant cell reports.* 11:618-622.

Gupta, J.N., and S.N. Gupta. 1995. Fertile plants regenerated from mesophyll protoplasts of cold tolerant rice. *Current Sci.* 68: 755-758

Hadley, H.H., and S.J. Openshaw. 1980. Interspecific and Intergeneric Hybridization. pp133-160. *In*: (eds). W. R. Fehr and H.H. Hadley. Hybridization of crop plants. American society of Agronomy and crop science society of America. Wisconsin, USA.

Hamrick J.L.; M. Jo Mary, and S.L. Susan. 1992. Factors influencing of genetic diversity in woody plant species. *New forests.* 6:95-124.

Hanks R.J.; G.L. Ashcroft, and V.P. Rasmussen. 1978. Corn production as influenced by irrigation and salinity Utah studies. *Irrig Sci.* 47:47-53.

Haq, N. 1996a. Molecular system: a tool to establish genetic diversity. Pp161-168. *In*: Quah, S.C.; R. Kiew; I. Bujang; M. Kusnan; N. Haq and P. de Groot (eds.). Underutilised Tropical Plant Genetic Resources: Conservation and Utilisation. Penerbit Universiti Pertanian, Malaysia.

Haq, N. 1996b. Gene transfer through wide hybridization. Pp207-218. *In*: Quah, S.C.; R. Kiew; I. Bujang; M. Kusnan; N. Haq and P. de Groot (eds.). Underutilised Tropical Plant Genetic Resources: Conservation and Utilisation. Penerbit Universiti Pertanian, Malaysia.

Harris, S.A.; C.E. Hughues; R.J. Abbott, and R. Ingram. 1994. Genetic variation in *Leucaena leucocephala* (Lam) de Wit. (Leguminosae: Mimosoideae). *Silvae Genetica.* 43(2-3):159-167

Hasegawa, S.; M. Thangaraj, and J.C. O'Toole. 1985. Root behaviour: field and laboratory studies for rice and non-rice crops. *In*: Soil physics and rice, IRRI, Los Banos, Philippines. pp.383-395.

Hedge D.M., and K. Srinivas. 1989. Irrigation and nitrogen fertility influences on plant water relations, biomass and nutrient accumulation and distribution in Banana cv. Robusta. *J. Hort. Sci.* 64: 89-95

Herdt, R.W. 1991. Research priorities for rice Biotechnology. pp19-54. *In: Rice Biotechnology.* (eds.). Khush, G.S. and G.H. Toenniessen. CAB International and International Rice Research Institute.

Herdt, R.W. 1996. Establishing the Rockefeller foundation's priorities for rice Biotechnology research in 1995 and beyond. pp17-30. *In: Rice Genetics III.* (Ed). Khush, G.S. International Rice Research Institute, Malina, Phillipines.

Heslop-Harrison, J.; Y. Heslop-Harrison, and K.R. Shivanna. 1984. The evaluation of pollen quality, and a further appraisal of the fluorochromatic (FCR) test procedure. *Theor. Appl. Genet.* 67:367-375.

Hodges, T.K; J.Peng; L.A. Lyznik, and D.S. Koetje. 1991. Transformation and regeneration of rice protoplasts. pp157-174. *In: Rice Biotechnology.* (eds.). Khush, G.S. and G.H. Toenniessen. CAB International and International Rice Research Institute (IRRI).

Holm, P.B.; S. Knudsen; P. Mouritzen; D. Negri; F.L. Olsen, and C. Roue. 1994. Regeneration of fertile barley plants from mechanically isolated protoplasts of fertilised egg cells. *The plant cell.* 6:531-543.

Hopkins, W.G. 1995. Introduction to plant physiology. John wiley & sons inc. New York, Chichester, Brisbane, Toronto and Singapore

Hossain, M. 1996. Economic prosperity in Asia: implications for rice research. *In*: Rice Genetics III. Proceedings of the Third International Rice Genetics Symposium, International Rice Research Institute. Manila (Philippines).pp. 3-16.

Hossain, M. 2001. Opening address: The challenge to feed the world's poor. *In*: Rice Biotechnology: Improving yield, stress tolerance and grain quality (eds.),A. Goode and D. Chadwick. Novartic Foundation. John Wiley & Sons Ltd. New York, Chichester, Brisbane, Singapore, Toronto pp. 1-10.

Howell, T.A., and E.A. Hiler. 1975. Optimization of water use efficiency under high frequency irrigation. I. Evaporation and yield relationship. *Transaction of the ASAE*. 18: 870-877.

Hsiao, T.C.; JC. O'Toole; E.B. Yambao, and N.C. Turner. 1984. Influence of osmotic adjustment on leaf rolling and tissue death in rice (*Oryza sativa* L.). *Plant Physiol.* 75:338-341.

Huang, B.Q., and S.D. Russell. 1992. Synergids degeneration in *Nicotiana*: a quantitative, flourochromatic and chlorotetracycline sudy. *Sex Plant reprod.* 5:151-155.

Ingram, K.T., and E. B. Yambao. 1988. Rice sensitivity to water deficit at different growth stages. *International Rice Research Newsletter*. 13(5):16-17.

Ingram, K.T.; J.G. Real; M.A. Maguling; M.A. Obien, and G.C. Loresto. 1990. Comparison of selection indices to screen lowland rice for drought resistance. *Euphytica*. 48:253-260.

Ingram, K.T.; F.D. Bueno; O.S. Namuco; E.B. Yambao, and C.A. Beyrouty. 1994. Rice root traits for drought resistance and their genetic variation. *In*: Rice roots: nutrient and water use (edited by Kirk, G.J.D.). International Rice Research Institute, Los Banos, Manila, Philippines. Pp.67-78.

IRRI. 1982. Drought resistance in crops with emphasis on rice. International Rice Research Institute, Los Banos, Manila, Philippines.

IRRI. 1982a. A plan for IRRI's third decade. International Rice Research Institute, Los Banos, Manila, Philippines.

IRRI. 1991. Rice germplasm, collecting, preservation and use. The International Rice Genebank. Genetic Resources Center, IRRI, P.O. Box 933, Manila, Philippines.

IRRI. 1995. Manual of operations and procedures of the International Rice Genebank. Genetic Resources Center, IRRI, Philippines.

IRRI. 1996. International Rice Research Institute. 1996. Rice Genetics III. Proceedings of the Third International Rice Genetics Symposium, 16-20 Oct 1995. IRRI, Manila, The Philippines.

IRRI. 1996a. Standard Evaluation System for Rice. International Rice Research Institute, Los Banos, Philippines.

Jain, R.K.; G. S. Khehra; S.H. Lee; N.W. Blackhall; R. Marcant; M.R. Power; E.C. Cocking, and S.S Gosal. 1995. An improved procedure for plant regeneration from indica and japonica rice protoplasts. *Plant cell Reports*. 14:515-519.

James, C. 2000. Transgenic crops worldwide: current situation and future outlook. *In*: Qaim, M., A.F. Krattiger and J. von Braun (eds.) Agricultural biotechnology in developing countries: Towards optimising the benefits for the poor. Kluwer Academic publications, Boston, Dordrecht, London. pp. 11-24.

Jana P.K., and A.M. Puste. 1985. Effect of soil moisture tension on yield, evapotranspiration and water use efficiency of winder maize. *Indian J. Agron.* 30:349-355.

Jearakongman, S.; S. Rajatasereekul; K. Naklang; P. Romyen; S. Fukai; E. Skulkhu; B. Jumpaket, and K. Nathabutra. 1995. Growth and grain yield of contrasting rice cultivars grown under different conditions of water availability. *Field crops research.* 44(2-3): 139-150.

Jenes, B., and J. Pauk. 1989. Plant regeneration from protoplast derived calli in rice (*Oryza sativa* L.). *Plant science*. 63: 187-198.

Jones, H.G. 1979. Visual estimation of plant water status in cereals. *J. Agric. Sci.* 92:83-89.

Jung, C. 2000. Molecular tools for plant breeding. *In*: Qaim, M., A.F. Krattiger and J. von Braun (eds.) Agricultural biotechnology in developing countries: Towards optimising the benefits for the poor. Kluwer Academic publications, Boston, Dordrecht, London. pp. 25-38.

Kalloo, G. 1992. Utilization of wild species. *In*: Hybridization of crop plants. Kalloo, G and JB. Chowdhury (eds) pp 149-171. Monograph, *Theor. Appl. Genet.* Vol. 16.

Kandasamy, S. 1981. Response of rice roots to simulated soil compaction with emphasis on water uptake. MS. Thesis, The University of Philippines at Los Banos, Philippines.

Karp, A. 1991. On the current understanding of somaclonal variation. *In*: Oxford surveys of plant molecular and cell biology, Vol. 7. (eds.), Miflin, B.J., Oxford University Press, pp1-58.

Khush, G.S. 1977. Disease and insect resistance in rice. *Advance in Agronomy.* 29:265-361.

Khush, G.S. 1984. *In*: Gustafson (ed.), Gene manipulation in plant improvement, Plenum, N.Y., 61-94.

Khush,G.S., and D.S. Brar. 1992. Overcoming the barriers in Hybridization. *In*: Hybridization of crop plants. Kalloo, G and JB. Chowdhury (eds) pp 47-61. Monograph, *Theor. Appl. Genet.* Vol. 16.

Khush, G.S. 1997. Origin, dispersal, cultivation and variation of rice. *Plant Mol. Biol.* 35:24-34.

Khush, G.S. 2001. Chair's introduction. *In*: Rice Biotechnology: Improving yield, stress tolerance and grain quality (eds.), A. Goode and D. Chadwick. Novartic Foundation. John Wiley & Sons Ltd. New York, Chichester, Brisbane, Singapore, Toronto pp. 11-12.

Kisaka, H.; M. Kisaka; A. Kanno, and T. Kameya. 1998. Intergeneric somatic hybridization of rice (*Oryza sativa* L.) and barley (*Hordeum vulgare* L.) by protoplast fusion. *Plant cell reports*. 17:362-367.

Kovacs, M.; B. Barnabas, and E. Kranz. 1995. Electro-fused isolated wheat (*Triticum aestivum* L.) gametes develop into multicellular structures. *Plant cell reports*. 15:178-180.

Koyama, M.L.; A. Levesley; R.M.D. Koebner; T.J. Flowers, and A.R. Yeo. 2001. Quantitative Trait Loci for component physiological traits determining salt tolerance in rice. *Plant Physiology*. 125: 406-422

Kramer, P.J. 1969. Plant and soil water relationships: A modern synthesis. McGraw-Hill, New York.

Kramer, P.J. 1980. Drought, stress and the origin of adaptation.pp7-20. *In*: Adaptation of plants to water and high temperature stress. (Eds.) Turner, N.C and P.J. Kramer. A Wiley interscience publication, New York, Chichester, Brisbane, Toronto.

Kranz, E.; J. Bautor, and H. Lorz. 1991a. *In vitro* fertilization of single, isolated gametes of maize mediated by electrofusion. *Sex plant reproduction*. 4:12-16.

Kranz, E.; J. Bautor, and H. Lorz. 1991b. Electrofusion mediated transmission of cytoplasmic organelles through the *in vitro* fertilization process, fusion of sperm cells with synergids and central cells and cell reconstitution in maize. *Sex plant reproduction*. 4:17-21.

Kranz, E., and H. Lorz. 1993. *In vitro* fertilization with isolated, single gametes results in zygotic embryogenesis and fertile maize plants. *The plant Cell*. 5:739-746.

Kranz, E., and H. Lorz. 1994. *In vitro* fertilization of maize by single egg and sperm cell protoplast fusion mediated by high calcium and high pH. *Zygote*, 2: 125-128

Kranz, E.; P. von Wiegen, and H. Lorz. 1995. Early cytological events after induction of cell division in egg cells and zygote development following *in vitro* fertilization with angiosperm gametes. *The plant journal*, 8: 9-23

Kranz, E., and T. Dresselhaus. 1996. *In vitro* fertilization with isolated higher plants gametes. *Tred. In plant science.* 1:82-89.

Kranz, E.; P. von Wiegen; H. Quader, and H. Lorz. 1998. Endosperm development after fusion of isolated, single maize sperm and central cells *in vitro. The plant Cell.* 10: 511-524.

Kranz, E., and J. Kumlehn. 1999. Angiosperm fertilization, embryo and endosperm development *in vitro. Plant Science.* 142:183-197.

Kumar, A., and Cocking, E.C. (1987). Protoplast fusion: A novel approach to organelle genetics in higher plant. *American Journal of Botany.* 74: 1289-1303.

Lee, L.; R.E. Schrol; H.D. Grimes, and T.K. Hodges. 1989. Plant regeneration from indica rice (*Oryza sativa* L.) protoplasts. *Planta.* 178:325-333.

Li, Z., and N. Murai. 1990. Efficiency plant regeneration from rice protoplasts in general medium. *Plant cell reports.* 9:216-220

Lilley, J.M.; MM Ludlow; S.R. McCouch; M.C. Champoux, and J.C. O'Toole. 1996. Location QTL for osmotic adjustment and dehydration tolerance in rice. *J. Expt. Bot.* 47:1427-1436.

Lopes, M.A., and B.A. Larkins. 1993. Endosperm origin, development and function. *The plant cell*: 5:1383-1399.

Loresto, G.C.; T.T. Chang, and O. Tagumpay. 1976. Field evaluation and breeding for drought resistance. *Philip. J. Crop Sci.* 1(1):36-39.

Ludlow, M.M.; F.J. Santamaria, and S. Fukai. 1990. Contribution of osmotic adjustment to grain yield of Sorghum bicolor (L) Moench under water limiting conditions. II. Water stress after anthesis. *Aust. J. Agric. Res.* 41: 67-78.

Ludlow, M.M., and RC Muchow. 1990. A critical evaluation of traits for improving crop yield in water limiting environments. *Adv. Argon.* 43: 107-153.

Ludlow, M.M.; J. Basnayake; M. Cooper; Y. Tao; R.C. Henzell; J.M. Manners; I.D. Godwin, and S.M.C Duncan. 1994. A Physiological genetic analysis of osmotic adjustment to water stress in grain sorghum. Pp 17-22. *In*: Use of molecular markers in Sorghum and Pearl Millet Breeding for developing countries (J.R. Witcombe and R.R. Duncan, eds.). ODA, England.

Lynch, P.T.; R.P Finch; M.R. Davey, and E.C. Cocking. 1991. Rice tissue culture and its application. pp135-156. *In: Rice Biotechnology.* (Eds.). Khush, G.S. and G.H. Toenniessen. CAB International and International Rice Research Institute (IRRI).

Lynch, J. 1995. Root architecture and plant productivity. *Plant Physiology.* 109: 7-13.

Mackill, D.J. 1996. Rainfed lowland rice improvement. International Rice Research Institute (IRRI), Los Banos, Manilla, Philippines.

Mannion, A.M. 1995. Agriculture and environmental change. Temporal and Spatial Dimensions. John Wiley & Sons Ltd, Chichester, New York, Brisbane, Toronto, Singapore.pp31-63, 304-341

Mansfield, T.A, and W.J. Davies. 1981. Stomata and stomata mechanisms. *In* L.G. Paleg and D. Aspinall (eds.). The physiology and Biochemistry of Drought resistance in plants. Academic press, Sydney, New York, London, Toronto, San Francisco. 315-346.

Matthews, R. B.; S.N. Azam-Ali, and J.M. Peacock. 1990. Response of 4 sorghum lines to mid-season drought. 2. Leaf characteristics. *Field crops research.* 25(3-4): 297-308.

Matthys-Rochon, E.; P. Vergne; S. Detechepare, and C. Dumas. 1987. Male germ unit isolation from three tricllular pollen species *Brassica oleracea, Zea mays* and *Triticum aestivum. Plant physiology.* 83: 464-466.

Matthys-Rochon, E.; C. Digonnet, and C. Dumas. 1992. Characterization of *Zea mays* embryo sac, using fluorescent probes and microinjection of Lucifer Yellow into the female cells. *In*:Bach, P.H., C.H. Reynolds, J.M. Clark, J. Mottley and P.L. Poole, (eds.). Biotechnology applications mircoinjection, Microscopic Imaging and Fluorescence. Plenum, New York. pp: 53-60.

Matthys-Rochon, E.; R. Mol; P. Heizmann, and C. Dumas. 1994. Isolation and microinjection of active sperm nuclei into egg cells and central cells of isolated maize embryo sacs. *Zygote.* 2: 29-35

Mezencev, N.; G. Clement, and E. Guiderdoni. 1995. Variation among progress of diploid plants regenerated from haploid microspore derived cell suspension protoplasts of rice (*Oryza sativa* L.). *Plant Breed.* 114:149-154.

Morgan, J.M. 1983. Osmoregulation as a selection criterion for drought tolerance in wheat. *Aust. J. Agric. Res.* 34:607-614.

Morgan, J.M.; R.A. Hare, and R.J. Fletcher. 1986. Genetic variation in osmoregulation in bread and durum wheats and its relationship to grain yield in range of field environments. *Aust. J. Agric. Res.* 37:449-457.

Marishima, H., and H.I. Oka. 1981. Phylogenetic differentiation of cultivated rice. 22. Numerical evaluation of indica japonica differentiation. *Japanese J. Breeding.* 31:402-413.

Mouemar, A., and J. Gasquez. 1983. Environmental conditions and isozyme polymorphism in *Chenopodium album* L. *Weed Research.* 23:141-149.

Neale, D.B.; M.E. Devey; K.D. Jermstad; M.R. Ahuja; M.C. Alosi, and K.A. Marshall. 1992. Use of DNA markers in forest tree improvement research. *New forests.* 6:391-407.

Nguyen, H.T., and C.P. Joshi. 1994. Molecular genetic approaches to improving heat and drought stress tolerance in crop plants. *In*: Biochemical and cellular mechanisms of stress tolerance in plants. J.H. Cherry (ed).pp.279-289.

Nguyen, H.T.; R.C. Babu, and A. Blum. 1997. Breeding for drought resistance in rice: physiology and molecular genetic considerations. *Crop Sci.* 37:1426-1434.

Nix, H.A. 1982. Aspects of drought from an agro-climatic perspective. *In*: Drought resistance in crops with emphasis on rice. International Rice Research Institute, Los Banos, Manila, Philippines. Pp. 3-15.

Noboru, E.; T. Ogawa, and G. S. Khush. 1997. Isozyme classification of Myanmar Rice Cultivars Resistant to Bacterial Blight. *Breeding Science*. 47:27-32.

Oka, H.I. 1991. Genetic Diversity of Wild and Cultivated Rice. pp 55-82. *In: Rice Biotechnology*. (Ed). Khush, G.S. and G.H. Toenniessen. CAB International and International Rice Research Institute.

Omara, L., and T.S.W. Maria. 2000. Isozyme variation in wild and cultivated species of the genus *Trifolium* L. (Leguminosae). *Annals of Botany*. 86:339-345.

O'Toole, J.C., and T.B. Moya. 1978. Genotypic variation in maintenance of leaf water potential in rice. *Crop Sci.* 18:873-876.

O'Toole, J.C., and T.T. Chang. 1979. Drought resistance in cereals-rice: a case study. *In*: Mussell & Staples (Eds.), Stress Physiology in Crop Plants. pp.373-405. John. Wiley & Sons, New York.

O'Toole, J.C., and R.T. Cruz. 1979. Response of leaf water potential, stomatal resistance and leaf rolling to water stress. *Plant Physiol.* 65: 428-432.

O'Toole, J.C., and M.A. Maguling. 1981. Greenhouse selection for drought resistance in rice. *Crop Sci.* 21:325-327.

O'Toole, J.C. 1982. Adaptation of rice to drought prone environments. *In*: Drought resistance in crops with emphasis on rice. International Rice Research Institute, Los Banos, Manila, Philippines. Pp.195-216

Pantuwan, G.; S. Fukai; M. Cooper; S. Rajatasereekul, and J.C. O'Toole. 2002. Yield response of rice (Oryza sativa L.) genotypes to different types of drought under rainfed lowlands Part 1. Grain yield and yield components. 2. Selection of drought resistant genotypes. 3. Plant factors contributing to drought resistance. *Field Crops Research*. 73: 153-200.

Paterson, A.H.; S. D. Tanksley, and M.E. Sorrells. 1991. DNA markers in plant improvement. *Adv. Agron*. 46:39-90.

Pathan, M.S. 1998. Identification of molecular markers linked to root penetration ability in rice (*Oryza sativa* L.). PhD Thesis, University of Texas Tech, USA.

Paudyal, K. P. 1999. Genetic diversity of Pummelo in Nepal and improvement of propagation methods. PhD Thesis. University of Southampton, UK.

Pisupati, B. 1999. Determination of genetic diversity among *porteresia coarctata* Tateoka accessions using morphology, isozyme and RAPD markers. *J. plant biochemistry and biotech.* 8:21-24.

Poehlman, J.M., and D.A. Sleper.1995. Rice breeding. *In*: Breeding of Field Crops. Iowa State University press, USA. Pp. 278-299.

Price, A.H.; A.D. Tomos, and D.S. Virk. 1997. Genetic dissection of root growth in rice (*Oryza sativa* L.).I: a hydroponic screen. *Theo. App. Genet.* 95: 132-143.

Price, A.H., and A.D. Tomos. 1997. Genetic dissection of root growth in rice (*Oryza sativa* L.).II: mapping quantitative trait loci using molecular markers. *Theo. App. Genet.* 95:143-152.

Quarrie, S.A., and H.G. Jones. 1979. Genotypic variations in leaf water potential, stomatal conductance and abscisic acid concentration in spring wheat subjected to artificial drought stress. *Ann. Bot.* 44:323-332

Ribaut, J.M.; D.A. Hoisington; J.A. Detsch; C. Jiang, and D. Gonzalez-de-Leon. 1996. Identification of quantitative trait loci under drought conditions in tropical maize. I. Flowering parameters and the anthesis-silking interval. *Theo. Appl. Genet.* 92:905-914.

Robertson, B.M.; A.E. Hall, and K.W. Foster. 1985. A field techniques for screening for genotypic differences in root growth. *Crop Science.* 25: 1084-1090.

Roeckel-Drevet; P. C. Digonnet; E. Matthys-Rochon; D. Champiat, and C. Dumas. 1995. Fertility of *Zea mays* pollen during dehydration: physiological steps outlined by nucleotide measurements. *Plant Physio. Biochem.* 33:289-294.

Rosenow, D.T.; J.E. Quiseberry; C.W. Wendt, and L.E. Clark. 1983. Drought tolerant sorghum and cotton germplasm. *Agron. Water Mgmt.* 7:207-222.

Rougier, M.; A.F. Antoine; D. Aldon, and C. Dumas. 1996. New lights in early steps of *in vitro* fertilization in plants. *Sex. plant reprod.* 9:324-329.

Russell, S.D. 1986. Isolation of sperm cells from pollen of *Plumbago zeylanica. Plant physiology.* 81:317-324.

Russell, S.D. 1992. Double fertilization. *International review of cytology.* 140:357-388.

Russell, S.D. 1993. The egg cell: Development and role in fertilization and early embryogenesis. *The plant cell.* 5:1349-1359.

Second, G. 1982. Origin of the genic diversity of cultivated rice (*Oryza* spp.): study of the polymorphism scored at 40 isozyme loci. *Japan J. Genet.* 57: 25-57.

Sharma, D.R.; R. Kaur, and K.Kumar. 1996. Embryo rescues in plants- a review. *Euphytica* 89: 325-337

Singh, D.P. 1989. Evaluation of specific dehydration tolerance traits for improvement of drought resistance. pp 165-175. *In*: Drought resistance in Cereals (Baker, F.W.G.,ed.). CAB international, Wallingford, UK.

Singh, Tej, and D.S. Malik. 1983. Effect of water stress at three growth stages on the yield and water use efficiency of dwarf wheat. *Irrigation Sci.* 4: 235-241.

Southworth, D. 1992. Freeze fracture of male reproductive cells. *International Review of cytology*. 140:187-204.

Spinosa, A.; D. Pignone, and G. Sonnate. 1998. Assessment of genetic variation in a working collection of *Vigna vexillata* (L). A rich by isozume and RAPD analaysis. *Genetic resources and crop evolution.* 45: 347-354.

Steponkus, P.L.; J.M. Cutler, and J.C. O'Toole. 1980. Adaptation to water stress in rice. 401-420. *In*: Adaptation of plants to water and high temperature stress. (Ed) Turner, N.C and P.J. Kramer. A willy interscience publication, New York, Chichester, Brisbane, Toronto.

Suh, S. C.; H.I. Kim, and W.Park. 1992. Plant regeneration from rice protoplasts culture in Javanica (cv Texmont.). *Biotechnol 1.* 34:1-9.

Suh, H.S.; Y.I. Sato, and H.Morishima. 1997. Genetic characterization of weedy rice (*Oryza sativa* L.) based on morpho-physiology, isozymes and RAPD markers. *Theor. Appl. Genet.* 94:316-321.

Sullivan, C.Y. 1971. Techniques for measuring plant drought stress. *In*: K.L. Larson, and J.D. Eastin (eds). Drought injury and resistance in crops. *Crop Sci. Soc. Am. Spec.* Pub. No. 2. Madison, Wis

Swindale, L.D., and F.R. Bidinger. 1981. Introduction: The human consequences of drought and crop research priorities for their alleviation. *In*: L.G. Paleg and D. Aspinall (eds). The physiology and Biochemistry of Drought resistance in plants. Academic press, Sydney, New York, London, Toronto, San Francisco. 15-38.

Tang, K.; X. Sun; D. An; J.B. Power; E. C. Cocking, and M.R. Davey. 2000. Plant regeneration from protoplasts of commercial Asian long-grain *javanica* rice. *Plant cell Tissue and Organ Culture.* 60 (1): 79-82

Tanksley, S.D.; N.D. Young; A.H. Paterson, and M.W. Bonierbale. 1989. RFLP mapping in plant breeding: New tools for an old science. *Biotechnology.* 7:257-264.

Tanksley, S.D., and S.R. McCouch. 1997. Seed banks and molecular maps: Unlooking genetic potential from the wild. *Science.* 27: 1063-1066.

Thangaraj, M.; J.C. O'Toole, and S.K. De Datta. 1990. Root responds to water stress in rainfed lowland rice. *Exp. Agric.* 26: 287-296.

Thanh, N.D.; H.G. Zheng; N.V. Dong; L.N. Trinh; M.L. Ali, and H.T. Nguyen. 1999. Genetic variation in root morphology and microsatellite DNA loci in upland rice (*Oryza sativa* L.) from Vietnam. *Euphytica.* 105:43-51.

Theunis, C.H., and J.L. Went.1989. Isolation of sperm cells from mature pollen grains of *Spinacia oleracea* L. *Sex. Plant Reprod.*2:97-102.

Theunis, C.H.; E.S. Pierson, and M. Cresti.1991. Isolation of male and female gametes in higher plants. *Sex. Plant Reprod.*4:145-154.

Theunis, C.H. 1992. Isolation and characterization of *Spinacia oleracea* L. sperm cells. PhD Thesis, van de Landbouwuniversiteit te Wageningen, The Netherlands.

Thompson, J.A.; R. Abdullah, and E.C. Cocking. 1986. Protoplast culture of rice (*Oryza sativa* L.) using media solidified with agarose. *Plant Science.* 47:123-133.

Tian H.Q., and S.D. Russell. 1997. Micromanipulation of male and female gametes of *Nicotiana tabacum*: I. Isolation of gametes. *Plant Cell Report.* 16: 555-560.

Tian H.Q., and S.D. Russell. 1997a. Micromanipulation of male and female gametes of *Nicotiana tabacum*: II. Preliminary attempts for *in vitro* fertilization and egg cell culture. *Plant Cell Report.* 16: 657-661.

Tian H.Q.; Z. Zhang, and S.D. Russell. 1998. Isolation of the male germ unit: organization and function in tobacco (*Nicotiana tabacum* L.). *Plant Cell Rep.* 18: 143-147.

Toenniessen, G.H. 1990. *In*: Higo, K. (ed.) 2nd International workshop on molecular biology of rice, Kawasaki Medical School, Okayama, p.39

Toenniessen, G.H. 1991. Potentially useful genes for rice genetic engineering.Pp. 253-280. *In*: Rice Biotechnolgy. ed. Khush and Toenniessen. CAB International, UK.

Tu, J.C., and C.S. Tan. 1991. Effect of soil compaction on growth, yield and root rots of white beans in clay loam and landy soil. *Soil Biol. Biochem.* 23:233-238.

Turner, N.C., and M.M. Jones. 1980. Turgor maintenance by osmotic adjustment: A review and evaluation.pp87-104. *In*: Adaptation of plants to water and high temperature stress. (Ed) Turner, N.C and P.J. Kramer. A Wiley interscience publication, New York, Chichester, Brisbane, Toronto.

Turner, F.T., and G.N. McCauley. 1983. Rice. *In*: I.D. Teare and M.M. Peet.(eds). Crop water relations. John Wiley and Sons Inc. New York, Chichester, Brisbane, Toronto, Singapore. pp 307-350.

Turner, N.C., and G.J. Burch. 1983. The role of water in plants. *In*: I.D. Teare and M.M. Peet.(eds). Crop water relations. John Wiley and Sons Insci. New York, Chichester, Brisbane, Toronto, Singapore. pp 73-126.

Turner N.C.; J.C. O'Toole; R.T. Cruz; E.B. Yambao; O.S. Namuco, and S. Ahmad. 1986. Response of seven diverse rice cultivars to water deficits. I. Stress development, canopy temperature, leaf rolling, and growth. *Field Crops Research.* 13: 257-271.

Turner N.C.; J.C. O'Toole; R.T. Cruz; E.B. Yambao; S. Ahmad; O.S. Namuco, and M. Dingkhun. 1986a. Response of seven diverse rice cultivars to water deficits.II. Osmotic adjustment, leaf elasticity, leaf extension, leaf death, stomatal conductance, and photosynthesis. *Field Crops Research.* 13: 273-286.

Turner, N.C.; G.C. Wright, and K.H.M. Siddique. 2001. Adaptation of grain legumes (Pulses) to water-limited environments. *Advances in Agronomy.* 71:193-232.

UNO. 2001. World Population Prospects, Population Division, Department of Economic and Social Affairs of United Nations, New York

var der Maas, H.M.; M.A.C.M. Zaal; E.R. de Jong; F.A. Krens, and J.L. van Went. 1993. Isolation of viable egg cells of perennial ryegrass (*Lolium perenne* L.). *Protoplasma.* 173: 86-89

var der Maas, H.M.; M.A.C.M. Zaal; E.R. de Jong; J.L. van Went, and F.A. Krens. 1993a. Optimization of isolation and storage of sperm cells from pollen of perennial ryegrass (*Lolium perenne* L.). *Sex Plant Reprod.* 6:64-70

var der Maas, H.M.; E.R. de Jong; A.C. Van Aelst; H.A. Verhoeven, and F.A. Krens. 1994. Cytological characterization of isolated sperm cells from pollen of perennial ryegrass (*Lolium perenne* L.). *Protoplasma.* 178: 48-56

Vaughan, D.A. 1994. The wild relatives of rice. IRRI, Manila, Philippines.

Virgona, J.M., and Barlow, E.W.R. 1991. Drought stress induces in the nonstructural carbohydrate composition of wheat stems. *Australian Journal of Plant Physiology.* Vol.18(3): 239-247

Virmani, S.S. 1996. Hybrid rice. *Advances in Agronomy.* 57: 377-462.

Volis, S.; M. Samuel; T. Yerlan; E. Usken; A. Saule, and N. Orlovsky. 2001. Allozyme variation in Turkmenian populations of wild barley, *Hordeum spontaneum* Koch. *Annals of Botany.* 87: 435-446.

Wagner, V.T.; Y.S. Song; E. Matthys-Rochon, and C. Dumas. 1990.Quantitive three-dimensional study on the position of the female gametoophyte and its constituent cells as a prerequisite for corn (*Zea mays* L.). *Theor. Appl. Genet.* 79:72-76.

Wang, R.L.; F.J. Wendel, and J.H. Dekker. 1995. Weedy adaptation in *Setaria spp.* Genetic diversity and population genetic structure in *S. gluca, S. geniculata* and *S. faberii* (Poaceae). *American Journal of Botany*. 82:1031-1039.

Weeden, N.F., and N.F. Wendel. 1990. Genetics of Plant Isozymes. *In*: (eds) Douglas E. Soltis and P. S. Soltis. Ioszyme in Plant Biology. Chapman and Hall, London. pp. 47-72.

Wendel, N.F., and N.F. Weeden. 1990. Visualization and Interpretation of Plant Isozymes. *In*: (eds) Douglas E. Soltis and P. S. Soltis. Ioszyme in Plant Biology. Chapman and Hall, London. pp. 5-46.

Wright, G.C.; R.C.G. Smith, and J.R. McWilliam. 1983. Differences between two grain Sorghum genotypes in adaptation to drought stress. I. Crop growth and yield responses. *Aust. J. Agric. Res.* 34: 615-626

Wright, P.J.; J.A. Green, and J.A. Callow. 1995. The focus sperm receptor for eggs. I. Development and characteristics of a binding assay. *J. Phycology*. 31:584-591

Yadav, R.; B. Courtois; N. Huang, and G. Mclaren. 1997. Mapping genes controlling root morphology and root distribution in a doubled haploid population of rice. *Theo. Appl. Genet.* 94:619-632.

Yamada, Y.; Y. Zhi-Qi, and D.T. Tang. 1986. Plant regeneration from protoplast-derived callus of rice (*Oryza sativa* L.). *Plant cell reports*. 5:85-88.

Yambao, E.B.; K. T. Ingram, and J. G. Real. 1992. Root xylem influence on the water relations and drought resistance of rice. *Journal of Experimental Botany*. 43(252): 925-932.

Yeo, A.R.; M.L. Koyama; S. Chinta, and T.J. Flowers. 2000. Salt Tokerance at the whole plant level. Pp107-124. *In*: (eds.) J. H. Cherry, R.D. Locy and A. Rychter. Plant Tolerance to abiotic stresses in Agriculture: Role of genetic engineering. Kluwer Academic Pulbishers, Dordrecht, Boston, London.

Yoshida, S. 1981. Fundamentals of rice crops science. International Rice Research Institute, Los Banos, Philippines.p.269.

Yoshida, S., and S. Hasegawa. 1982. The rice root system: its development and function. *In*: Drought resistance in crops with emphasis on rice. International Rice Research Institute, Los Banos, Philippines. pp.97-114.

Yu, R., and W.A. Jensen. 1985. Ultrastructural observations of the mature megagametophyte and the fertilization in wheat (*Triticum aestivum*). *Can. J. Bot.* 63: 163-178

Yu, L.; J.D. Ray; J.C. O'Toole, and H.T. Nguyen. 1995. Use of wax-petrolatum layers for screening rice root penetration. *Crop Sci.* 35:684-688.

Zhang, G.; M.K. Campenot; L.E. McGann, and D.D. Cass. 1992. Flow cytometric characteristics of sperm cells isolation from pollen of *Zea mays* L. *Plant physiology*. 99:54-59.

Zhang, G.; C.M. Williams; M.K. Campenot; L.E. McGann; L.E. Cutler, and D.D. Cass. 1995. Effect of calcium, magnesium, potassium and boron on sperm cells isolation from pollen of *Zea mays* L. *Sex. Plant Reprod.* 8:113-122.

Appendices

Appendix: Table 3.1 Mean and one standard deviation of days to seedlings emergence, tiller number per plant, drought score, ET (mm/day), WUE (g/kg) of rice accessions in experiment-1

Name of Accessions	Days to emergence	Tiller/plant	Visual drought score	ET (mm/day)	WUE (g/kg)
Keora	10.50±1.91cde	4.18±1.52abc	1.56±0.43a	3.43±0.36	1.78±0.15
Dhapa	14.25±0.96ghi	6.96±1.53de	1.63±0.63a	4.32±0.60	2.04±0.39
Hogla	15.25±2.63hi	4.70±0.72abc	1.63±0.48a	4.09±0.16	1.57±0.28
Tilock Kachari	14.25±0.96ghi	4.67±1.92abc	1.69±0.75a	4.41±0.43	1.98±0.16
Kala Mona	14.25±1.50ghi	4.66±1.25abc	1.75±0.29a	3.93±0.45	1.99±0.23
Dud Kalam	13.00±1.41fg	7.88±1.32e	1.75±0.29a	3.38±0.34	2.15±0.13
Kumari	15.00±2.89I	5.63±1.97bcd	2.38±1.44ab	4.06±0.49	1.87±0.45
Aswina	10.75±1.50cde	4.68±0.49abc	2.63±0.48abc	4.73±0.15	1.45±0.15
Kartik Sail	9.00±2.00bc	4.39±0.28abc	2.75±0.29a-d	3.67±0.45	1.68±0.38
Raja Sail	12.00±0.82efg	4.57±1.00abc	2.88±0.75a-d	4.19±0.29	1.99±0.36
Kada Moni	11.75±0.50def	3.62±0.43a	3.00±1.15a-d	3.28±0.35	2.08±0.28
Dud Mona	9.75±0.96bcde	4.49±1.04abc	3.25±2.06a-d	4.16±0.26	1.96±0.27
Kajal Sail	12.00±1.82efg	5.72±1.18cd	3.50±2.04a-d	4.25±0.36	1.77±0.27
Kola Mocha	8.75±0.50abc	4.10±0.48abc	3.63±1.60a-d	3.75±0.19	1.54±0.20
Hogla Pata	6.50±0.58a	5.72±0.77cd	3.75±1.32a-d	3.75±0.27	2.07±0.06
Panbira-2	9.75±0.50bcde	5.13±1.13abc	4.63±2.52bcd	4.08±0.28	1.85±0.12
Nuncha	7.75±1.26ab	4.44±0.40abc	4.63±2.25bcd	4.18±0.17	1.66±0.09
Nona Balam	9.00±1.41bc	4.33±0.91abc	4.88±2.66cd	3.71±0.21	1.75±0.29
Kacha Mota	9.50±0.58bcd	4.75±0.54abc	5.00±1.78cd	3.96±0.18	1.90±0.14
Lakhai	9.50±1.29bcd	3.80±1.15ab	5.25±1.94d	3.93±0.18	1.83±0.49
Kumra Gair	8.00±2.00ab	5.24±0.67a-d	7.88±0.95e	4.74±0.14	1.87±0.28
SE	0.32	0.15	0.22	0.08	0.02
DMRT*	0.79/0.65	0.37/0.30	0.54/0.44	0.24/0.15	0.05/0.04

SE= Standard error of mean, Mean followed by alphabet showing DMRT ranking: Any two means having a common letter is not significantly different at the 5% level,
*Largest/smallest shortage significant range values

Appendix: Table 3.2 Mean and one standard deviation of shoot length (cm), root length (cm), root-shoot length ratio, shoot dry weight (g) per plant, root dry weight (g) per plant, root-shoot dry weight ratio, total dry weight (g) per plant of rice accessions in experiment-1

Name of accessions	Shoot length (cm)	Root length (cm)	Root-to-shoot length ratio	Root dry mass (g) per pot	Shoot dry mass (g) per pot
Keora	58.61±2.67c-f	40.21±1.94e	0.69±0.07e	2.56±0.29	6.22±1.46
Dhapa	56.37±2.47bcd	36.41±6.25de	0.64±0.11de	2.68±0.43	9.31±3.43
Hogla	59.56±1.62def	27.37±3.57bc	0.46±0.06ab	1.28±0.39	6.11±1.25
Tilock Kachari	58.98±1.38c-f	40.41±3.87e	0.69±0.07e	2.30±0.25	7.70±1.94
Kala Mona	63.18±3.85ef	42.62±2.48f	0.68±0.11e	2.70±0.28	8.03±1.59
Dud Kalam	56.32±3.15bcd	36.25±4.24de	0.64±0.02de	2.06±0.53	11.19±2.48
Kumari	58.32±3.43c-f	27.35±2.86bc	0.48±0.10abc	1.49±0.09	9.52±5.27
Aswina	52.18±1.71abc	31.75±2.50c	0.61±0.07cde	1.70±0.29	6.52±0.26
Kartik Sail	49.69±2.94ab	31.66±5.58c	0.65±0.13de	1.75±0.47	6.95±2.74
Raja Sail	52.25±1.24abc	34.25±7.10d	0.65±0.11de	1.90±0.41	8.79±2.33
Kada Moni	53.00±2.48a-d	39.09±2.13e	0.74±0.03e	2.70±0.37	6.21±0.66
Dud Mona	60.72±1.89ef	31.99±8.76c	0.53±0.15bcd	1.88±0.25	10.60±3.02
Kajal Sail	51.89±2.80abc	19.61±2.30a	0.38±0.04a	1.41±0.89	7.68±1.08
Kola Mocha	56.37±2.82bcd	26.42±4.26b	0.47±0.11abc	1.24±0.09	5.96±0.17
Hogla Pata	48.31±0.72a	25.70±4.63b	0.53±0.08bcd	1.86±0.41	8.25±1.08
Panbira-2	55.94±2.03b-e	19.08±4.30a	0.35±0.10a	1.23±0.61	7.93±0.62
Nuncha	53.61±2.47a-d	26.15±3.80b	0.49±0.08abc	1.15±0.18	6.83±0.51
Nona Balam	56.44±1.75b-e	20.01±5.59ab	0.35±0.09a	1.64±0.62	6.18±1.83
Kacha Mota	63.08±1.36ef	23.49±4.73ab	0.37±0.07a	1.80±0.48	9.47±1.37
Lakhai	64.76±2.28f	23.14±4.83ab	0.36±0.09a	1.58±1.19	6.55±1.33
Kumra Gair	57.89±1.89c-f	23.45±6.03ab	0.41±0.10ab	1.60±0.85	8.46±2.17
SE	0.65	0.91	0.11		
DMRT*	1.60/1.29	2.24/1.82	0.27/0.22		

SE= Standard error of mean, Mean followed by alphabet showing DMRT ranking: Any two means having a common letter are not significantly different at the 5% level, *Largest/smallest shortage significant range values

Appendix: Table 3.2 Cont.

Name of accessions	Drought Score	Shoot dry weight (g)	Root dry weight (g)	Root-to-shoot dry weight ratio	Total dry weight (g)
Keora	1.56±0.43a	1.25±0.29a	0.51±0.06de	0.42±0.10de	1.76±0.30b
Dhapa	1.63±0.63a	1.86±0.69ab	0.53±0.09e	0.31±0.05bcd	2.39±0.77cd
Hogla	1.63±0.48a	1.23±0.25a	0.26±0.08ab	0.21±0.06abc	1.49±0.26a
Tilock Kachari	1.69±0.75a	1.54±0.39ab	0.46±0.05de	0.31±0.05bcd	2.00±0.43bc
Kala Mona	1.75±0.29a	1.61±0.32ab	0.54±0.06e	0.35±0.08cde	2.15±0.30c
Dud Kalam	1.75±0.29a	2.24±0.50b	0.47±0.11de	0.19±0.07ab	2.71±0.46e
Kumari	2.38±1.44ab	1.91±1.05ab	0.30±0.02b	0.19±0.05ab	2.20±1.07c
Aswina	2.63±0.48abc	1.31±0.05a	0.34±0.05c	0.26±0.05abc	1.65±0.06b
Kartik Sail	2.75±0.29a-d	1.39±0.55a	0.35±0.09c	0.26±0.06abc	1.74±0.62b
Raja Sail	2.88±0.75a-d	1.76±0.47ab	0.41±0.08d	0.23±0.08abc	2.14±0.47c
Kada Moni	3.00±1.15a-d	1.24±0.13a	0.54±0.07e	0.44±0.04e	1.78±0.19b
Dud Mona	3.25±2.06a-d	2.12±0.61b	0.37±0.05d	0.19±0.05ab	2.49±0.62d
Kajal Sail	3.50±2.04a-d	1.54±0.22ab	0.28±0.17b	0.18±0.09ab	1.82±0.39b
Kola Mocha	3.63±1.60a-d	1.19±0.04a	0.25±0.02ab	0.21±0.02abc	1.44±0.02a
Hogla Pata	3.75±1.32a-d	1.65±0.22ab	0.37±0.08d	0.23±0.03abc	2.02±0.29bc
Panbira-2	4.63±2.52bcd	1.59±0.12ab	0.25±0.12ab	0.15±0.07a	1.83±0.21b
Nuncha	4.63±2.25bcd	1.37±0.10a	0.23±0.04a	0.17±0.04ab	1.60±0.09ab
Nona Balam	4.88±2.66cd	1.24±0.37a	0.33±0.12bc	0.28±0.12abc	1.57±0.40ab
Kacha Mota	5.00±1.78cd	1.90±0.27ab	0.36±0.10c	0.19±0.05ab	2.26±0.31c
Lakhai	5.25±1.94d	1.31±0.27a	0.32±0.24bc	0.25±0.20abc	1.63±0.34ab
Kumra Gair	7.88±0.95e	1.69±0.44ab	0.32±0.17bc	0.20±0.14ab	2.01±0.46bc
SE	0.22	0.06	0.02	0.01	0.07
DMRT*	0.54/0.44	0.15/0.12	0.05/0.04	0.03/0.02	0.17/0.14

SE= Standard error of mean, Mean followed by alphabet showing DMRT ranking: Any two means having a common letter are not significantly different at the 5% level,
*Largest/smallest shortage significant range values

Appendix: Table 3.3 Mean and one standard deviation of leaf length (cm), leaf width (cm), leaf area (cm^2), stomata number (per mm^2) and stomata conductance (s/cm) before and at stress conditions of rice accessions in experiment-1

Name of accession	Drought Score	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)
Keora	1.56±0.43a	39.95±1.75d-g	0.93±0.04g	21.74±1.89g
Dhapa	1.63±0.63a	37.15±4.05a-e	0.90±0.09g	19.66±3.88c
Hogla	1.63±0.48a	38.95±3.13b-f	0.89±0.09fg	20.36±3.75e
Tilock Kachari	1.69±0.75a	38.47±2.07a-e	0.78±0.03b-e	17.65±0.74bc
Kala Mona	1.75±0.29a	40.61±2.30efg	0.86±0.04efg	20.38±1.77e
Dud Kalam	1.75±0.29a	40.44±2.41efg	0.76±0.05bcd	18.07±1.22bc
Kumari	2.38±1.44ab	36.99±3.99a-e	0.78±0.02b-e	16.92±2.22b
Aswina	2.63±0.48abc	35.89±1.83a-d	0.71±0.02ab	14.92±1.17ab
Kartik Sail	2.75±0.29a-d	8.47±4.09abc	0.76±0.10bcd	15.82±3.49b
Raja Sail	2.88±0.75a-d	37.33±1.84a-e	0.76±0.04bcd	16.54±1.31b
Kada Moni	3.00±1.15a-d	35.79±1.68a-d	1.02±0.02h	21.39±1.16f
Dud Mona	3.25±2.06a-d	39.93±1.35d-g	0.81±0.01def	19.04±0.74bc
Kajal Sail	3.50±2.04a-d	34.86±1.93ab	0.72±0.05abc	14.81±1.77ab
Kola Mocha	3.63±1.60a-d	42.20±3.74fg	0.78±0.03b-e	19.19±2.26c
Hogla Pata	3.75±1.32a-d	34.45±2.09a	0.67±0.02a	13.62±1.08a
Panbira-2	4.63±2.52bcd	39.41±2.49c-f	0.74±0.04a-d	17.12±1.87bc
Nuncha	4.63±2.25bcd	38.30±3.04a-f	0.80±0.04cde	17.91±2.15bc
Nona Balam	4.88±2.66cd	37.82±2.13a-f	0.70±0.04ab	15.50±1.45ab
Kacha Mota	5.00±1.78cd	44.27±2.36g	0.74±0.05a-d	19.09±1.93c
Lakhai	5.25±1.94d	42.05±2.89fg	0.91±0.08g	22.25±1.50g
Kumra Gair	7.88±0.95e	40.87±2.20efg	0.85±0.03efg	20.35±1.72e
SE	0.22	0.38	0.01	0.36
DMRT*		0.91/0.76	0.03/0.02	0.88/0.72

SE= Standard error of mean, Mean followed by alphabet showing DMRT ranking: Any two means having a common letter are not significantly different at the 5% level,
*Largest/smallest shortage significant range values

Appendix: Table 3.3 Cont.

Name of accession	Drought score	Leaf stomata number (per mm^2)	Stomata conductance before stress (cm/s)	Stomata conductance at stress (cm/s)
Keora	1.56±0.43a	134±12abc	0.38±0.05e	0.05±0.006b
Dhapa	1.63±0.63a	148±15abc	0.34±0.08c	0.04±0.01a
Hogla	1.63±0.48a	125±11abc	0.24±0.05b	0.07±0.007c
Tilock Kachari	1.69±0.75a	150±41abc	0.40±0.13e	0.06±0.01b
Kala Mona	1.75±0.29a	142±11abc	0.35±0.02de	0.06±0.01b
Dud Kalam	1.75±0.29a	139±48abc	0.38±0.03e	0.04±0.01b
Kumari	2.38±1.44ab	153±26abc	0.36±0.10e	0.06±0.03b
Aswina	2.63±0.48abc	117±15a	0.30±0.05b	0.08±0.01c
Kartik Sail	2.75±0.29a-d	123±24abc	0.28±0.04b	0.06±0.008b
Raja Sail	2.88±0.75a-d	126±25abc	0.24±0.05b	0.07±0.01c
Kada Moni	3.00±1.15a-d	135±20abc	0.37±0.07e	0.04±0.005b
Dud Mona	3.25±2.06a-d	124±15abc	0.27±0.05b	0.04±0.01b
Kajal Sail	3.50±2.04a-d	141±18abc	0.27±0.04b	0.06±0.01b
Kola Mocha	3.63±1.60a-d	132±8abc	0.19±0.03a	0.05±0.02b
Hogla Pata	3.75±1.32a-d	159±24e	0.37±0.09e	0.07±0.03c
Panbira-2	4.63±2.52bcd	131±12abc	0.26±0.06b	0.07±0.02c
Nuncha	4.63±2.25bcd	134±25abc	0.25±0.06b	0.08±0.02c
Nona Balam	4.88±2.66cd	157±14bc	0.34±0.04d	0.08±0.007c
Kacha Mota	5.00±1.78cd	120±29ab	0.20±0.02a	0.06±0.02b
Lakhai	5.25±1.94d	131±13abc	0.34±0.07c	0.07±0.03c
Kumra Gair	7.88±0.95e	121±4ab	0.21±0.02a	0.08±0.01c
SE	0.22	3.00	0.01	0.002
DMRT	0.54/0.44	7.08/5.77	0.03/0.02	0.005/0.004

SE= Standard error of mean, Mean followed by alphabet showing DMRT ranking: Any two means having a common letter are not significantly different at the 5% level,
*Largest/smallest shortage significant range values

Appendix: Table 3.4 Regression coefficients of water use efficiency (g/kg) (dependent variable) to independent variables of total dry weight (g) per plant and evapotranspiration in experiment-1

Parameters	Un-standardized coefficients		Standardized coefficients	t	Sig.
	B	Std Error	Beta		
Constant	1.942	0.069		28.09	0.000
Total dry weight (g) per plant	0.86	0.04	1.46	22.98	0.000
Evapotranspiration (mm/day)	-0.45	0.027	-1.05	-16.58	0.000

Sig. = Significant level, t= student test

Appendix: Table 3.4a Regression model for water use efficiency in experiment-1

Model	R	R^2	Adjusted R^2	Est. std error
	0.93	0.87	0.87	0.11

Appendix: Table 3.4b Analysis of variance for regression model of dependent variable water use efficiency in experiment-1

Source of variation	Sum of square	Df	Mean square	F	Sig.
Regression	6.64	2	3.32	265.59	0.000
Residual	1.01	81	0.01		
Total	7.66	83			

D.f.= Degree of freedom

Appendix: Table 3.5 Regression coefficients of ET (dependent variable) to independent variables of leaf length (cm), leaf width (cm), leaf area (cm^2), stomata number (per mm^2), stomata conductance (cm/s) at and before stress, water use efficiency, total dry mass (g) per plant and tiller number per plant in experiment-1

Parameters	Un-standardized coefficients		Standardized coefficients	t	Sig.
	B	Std Error	Beta		
Constant	3.99	0.082		48.66	000
Root dry weight (g) per plant	1.45	0.25	0.27	5.86	0.000
Root-shoot dry weight ratio	-1.41	0.299	-0.204	-4.712	0.000
Total biomass (g) per plant	1.582	0.045	1.155	35.02	000
Water use efficiency (g/kg)	-1.847	0.049	-0.80	-37.57	000

Sig. = Significant level, t= student test

Appendix: Table 3.5a Regression model for dependent variable of ET in experiment-1

Model	R	R^2	Adjusted R^2	Est. std error
	0.99	0.98	0.98	0.099

Appendix: Table 3.5b Analysis of variance for regression model of dependent variable ET in experiment-1

Source of variation	Sum of square	Df	Mean square	F	Sig.
Regression	40.62	4	10.156	1035.366	0.000
Residual	0.775	79	0.01		
Total	41.40	83			

D.f.= Degree of freedom

Appendix: Table 3.6 Different principal components and their variance (%) extracted by principal component analysis on the basis of physio-morphological characteristics in experiment-1

Principal Components	Initial Eigen values			Extraction sums of squared loadings		
	Total	% of variance	Cumulative %	Total	% of Variance	Cumulative %
1	6.82	35.92	35.92	6.82	35.92	35.92
2	4.49	23.65	59.56	4.49	23.65	59.56
3	3.00	15.79	75.36	3.00	15.79	75.36
4	1.36	7.14	82.49	1.36	7.14	82.49
5	0.86	4.51	87.00			
6	0.75	3.92	90.93			
7	0.61	3.22	94.15			
8	0.35	1.82	95.96			
9	0.27	1.45	97.41			
10	0.23	1.20	98.62			

Appendix: Table 3.7 Mean and one standard deviation of days to emergence, tiller number per plant, drought score, ET (mm/day) and WUE (g/kg) of different accessions of rice in experiment-2

[a]Name of accessions	Days to emergence	Tiller number/plant	Drought score	ET (mm/day)	WUE (g/kg)
Dud Kalam	14.25±0.98fgh	8.87±2.84b-e	1.56±0.72a	3.12±0.05a-e	6.94±0.17a-i
Aug Meghi	14.63±0.48ghi	4.93±1.57ab	1.63±0.63a	3.07±0.10a-e	6.97±0.49a-i
Keora	11.50±1.12abc	5.18±1.69abc	1.75±0.50a	3.03±0.07a-e	6.58±0.70a-h
Manik Mondal	12.00±0.82bcd	3.90±0.50a	1.75±0.50a	3.05±0.11a-e	6.27±0.45a-f
Huma gambir	13.00±0.82def	6.73±1.78a-d	1.75±0.96a	3.04±0.11a-e	6.98±0.95a-f
Agali	14.75±0.50ghi	10.93±1.88def	1.75±0.50a	3.09±0.15a-e	6.74±0.71a-i
Kola Mocha	11.75±1.50bcd	8.28±2.35a-e	1.75±0.96a	3.05±0.08a-e	6.91±0.76a-i
Dhapa	15.25±0.68hi	7.96±3.46a-e	1.75±0.25a	2.91±0.17a	7.14±0.66a-j
Bina Muri-2	12.50±0.58b-e	6.88±0.38a-d	1.75±0.50a	3.02±0.09bcde	5.67±0.20a-d
Nona Balam	11.63±0.95bcd	6.03±0.84abc	1.88±0.48ab	3.13±0.11a-e	7.98±1.88a-k
Kala Mona	14.00±0.82fgh	9.25±4.09b-e	1.88±0.75ab	3.03±0.09a-e	7.64±0.54a-k
Kada Moni	14.13±0.63fgh	6.10±0.36abc	1.90±0.49ab	3.04±0.10a-e	6.54±0.87a-h
Bina Muri-1	12.50± 0.58b-e	6.33±0.97a-d	2.00±0.58ab	2.96±0.13abc	5.77±0.32a-e
Hogla Pata	10.75±0.96a	7.55±1.32a-e	2.00±0.41ab	2.96±0.15abcd	6.40±0.26a-g
Bali Guri	14.75±0.50ghi	17.35±11.62h	2.12±3.48ab	3.12±0.21a-e	7.55±1.81a-k
Aus Nagra	14.00±0.82fgh	5.60±2.25abc	2.25±0.50ab	3.10±0.04a-e	7.25±1.20a-j
Hazi Faram	14.13±0.25f-I	9.18±7.24b-e	2.25±0.50ab	3.17±0.07de	6.68±0.30a-i
Tilock Kachari	15.38±1.11hi	13.55±5.75fgh	2.25±0.50ab	3.07±0.08a-e	7.17±0.83a-j
Bakee	14.75±0.50ghi	5.40±0.98abc	2.25±0.50ab	3.12±0.08a-e	7.03±0.73a-I
Lakhai	11.00±0.82ab	4.73±0.17ab	2.50±0.82b	3.02±0.12a-e	5.55±0.77abc
Baila Bokri	14.75±0.50ghi	6.20±2.25a-d	2.50±1.15b	3.20±0.05e	8.35±2.35h-k
Kumri Aus	14.75±0.50ghi	11.83±6.28efg	2.50±1.41b	3.19±0.09e	9.17±2.49k
Hasha	14.00±0.82fgh	15.75±4.76gh	2.50±0.96b	3.17±0.29de	9.11±2.55k
Aus Kushi	14.25±0.87f-I	8.95±2.27a-e	2.75±0.96bc	3.15±0.05cde	7.70±1.27a-k
Dular	11.50±0.58abc	5.90±1.07abc	2.75±0.96bc	2.94±0.16abc	6.96±1.32bc
Hashi Kalmi	11.63±0.48bcd	5.98±1.62abc	2.75±1.26bc	2.97±0.10abcd	6.67±0.54bc
Hanumanjata	11.00±0.82ab	4.98±0.51ab	2.75±0.96bc	3.12±0.17a-e	5.86±1.10a-e
Nuncha	10.75±1.50a	5.90±1.36abc	2.75±1.25bc	3.10±0.11a-e	6.58±1.01a-h
Panbira-1	12.13±1.03b-e	5.15±0.76abc	2.75±1.25bc	3.10±0.08a-e	6.47±0.62abc
Kartik Sail	11.38±1.25abc	6.63±0.62a-d	3.00±0.58c	3.02±0.08a-e	6.43±0.52a-g
Panbira-2	11.50±0.58bcd	7.68±1.69a-e	3.00±0.58c	3.14±0.20cde	6.95±0.90a-i
Bogi	12.00±0.82bcd	6.48±0.37a-d	3.00±0.58c	2.92±0.20ab	5.38±1.47ab
Bok Tulsi	12.50±0.58b-e	5.35±0.49abc	3.00±1.29c	3.00±0.09a-e	6.88±0.91a-I
Binna Toa	12.00±0.82bcd	7.88±1.29a-e	3.25±0.50cd	3.14±0.14cde	7.48±0.94a-k
Hogla	15.25±0.50hi	5.70±2.64abc	3.25±0.96cd	3.01±0.15a-e	6.26±0.42a-g
Kumari	15.75±1.25j	6.63±1.26a-d	3.50±0.98d	3.04±0.10a-e	6.74±0.80a-i
Hijolee	11.25±0.96ab	6.53±0.69a-d	3.50±1.41d	3.06±0.07a-e	6.66±0.53bc
Marichbati	11.75±1.50bcd	5.98±1.81abc	3.50±1.15d	3.10±0.08a-e	7.44±1.04bcd
Sada Aus	14.63±0.48ghi	9.85±1.31c-f	3.50±0.82d	3.14±0.14cde	8.84±1.29jk
Gopal bhog	13.50±0.58efg	5.78±1.21abc	3.75±1.50de	3.19±0.10e	6.38±0.88a-g
Aswina	13.75±0.25efg	5.68±1.58abc	3.75±0.96de	3.20±0.11e	6.98±0.91a-i
Kataktara	12.13±1.18b-e	4.75±0.65ab	4.25±0.50e	3.04±0.15a-e	6.26±0.83a-f
Aus Baku	12.75±0.50cde	4.68±.0.26ab	4.50±1.41e	3.13±0.10bcde	6.16±0.69a-f
Ausa Bogi	11.50±1.29efg	6.40±0.82a-d	4.75±0.96ef	3.00±0.07a-e	5.35±0.56a
Kacha Mota	12.38±0.48b-e	7.98±0.59a-e	4.75±1.25ef	3.06±0.08a-e	7.27±0.69a-j
Boalia	13.50±1.29efg	6.13±0.74a-d	5.00±1.29ef	3.01±0.10a-e	8.50±0.20ijk
Dharial	11.13±0.25ab	9.05±1.77b-e	5.75±1.26f	3.09±0.09a-e	6.92±1.37bc
Kumra Gair	11.50±1.00abc	7.70±1.48a-e	6.00±1.29f	3.09±0.04a-e	7.20±1.09a-j
SE	0.12	0.26	0.10	0.01	0.10
DMRT*	0.36/0.24	0.77/0.51	0.29/0.19	0.03/0.02	0.29/0.19

[a]Accessions in bold letters were also used in experiment-1, SE= Standard error of mean, Mean followed by alphabet showing DMRT ranking: Any two means having a common letter are not significantly different at the 5% level, *Largest/smallest shortage significant range values

Appendix: Table 3.8 Mean and one standard deviation of root length (cm), shoot length (cm) and root-shoot length ratio of rice accessions in experiment-2

[a]Name of accessions	Drought Score	Root length (cm)	Shoot length (cm)	Root-shoot length ratio
Dud Kalam	1.56±0.72a	33.58±2.78jk	63.32±2.58b-e	0.53±0.08k
Aug Meghi	1.63±0.63a	30.87±3.25g-k	78.43±1.52j-p	0.39±0.04b-h
Keora	1.75±0.50a	29.97±1.96f-k	65.89±3.58b-g	0.45±0.06h-k
Manik Mondal	1.75±0.50a	30.25±1.98f-k	74.55±3.23g-m	0.41±0.04d-i
Huma gambir	1.75±0.96a	30.42±1.68f-k	76.93±9.29h-n	0.40±0.05c-i
Agali	1.75±0.50a	29.08±4.41d-k	79.95±6.43l-p	0.36±0.03a-h
Kola Mocha	1.75±0.96a	23.57±1.97a-f	73.05±4.23f-m	0.32±0.04a-f
Dhapa	1.75±0.25a	34.12±2.25k	68.76±2.49b-i	0.50±0.10ijk
Bina Muri-2	1.75±0.50a	27.28±4.96a-h	60.90±3.13b	0.45±0.08f-j
Nona Balam	1.88±0.48ab	28.46±1.67c-k	70.83±3.42d-l	0.40±0.02d-h
Kala Mona	1.88±0.75ab	34.10±2.79k	84.48±3.94nop	0.40±0.02b-h
Kada Moni	1.90±0.49ab	32.90±1.94ijk	71.75±4.32e-l	0.46±0.03h-k
Bina Muri-1	2.00±0.58ab	26.55±5.14a-h	61.25±5.39bc	0.43±0.05e-j
Hogla Pata	2.00±0.41ab	27.55±2.61b-k	69.20±2.48b-j	0.40±0.02b-h
Bali Guri	2.12±3.48ab	24.25±3.18a-g	52.80±3.60a	0.46±0.03h-k
Aus Nagra	2.25±0.50ab	25.58±3.13a-h	86.08±7.17p	0.30±0.02ab
Hazi Faram	2.25±0.50ab	24.05±3.63a-f	85.73±6.99op	0.28±0.03a
Tilock Kachari	2.25±0.50ab	31.22±3.99h-k	61.90±3.51bcd	0.50±0.07jk
Bakee	2.25±0.50ab	21.73±1.77abc	79.25±1.47k-p	0.27±0.02a
Lakhai	2.50±0.82b	21.88±3.10abc	71.58±6.62e-l	0.31±0.03a-d
Baila Bokri	2.50±1.15b	25.98±2.24a-h	81.68±8.34m-p	0.32±0.04a-f
Kumri Aus	2.50±1.41b	25.08±5.13a-h	77.77±3.45i-p	0.32±0.05a-f
Hasha	2.50±0.96b	25.75±5.32a-h	76.43±4.21h-n	0.34±0.09a-g
Aus Kushi	2.75±0.96bc	21.98±2.92abc	67.78±6.67b-h	0.32±0.03a-e
Dular	2.75±0.96bc	30.33±3.38f-k	71.25±5.35e-l	0.43±0.04g-j
Hashi Kalmi	2.75±1.26bc	29.35±2.76e-k	70.18±3.12c-k	0.42±0.03f-j
Hanumanjata	2.75±0.96bc	25.40±7.83a-h	69.83±4.02b-j	0.36±0.09a-h
Nuncha	2.75±1.25bc	20.63±0.75a	72.10±1.54e-l	0.29±0.001a
Panbira-1	2.75±1.25bc	22.03±3.80abc	70.78±3.66d-l	0.31±0.06a-d
Kartik Sail	3.00±0.58c	21.40±4.34ab	71.95±0.98e-l	0.30±0.06ab
Panbira-2	3.00±0.58c	22.95±6.60a-e	65.40±4.74b-g	0.36±0.08g-j
Bogi	3.00±0.58c	21.95±2.72abc	71.68±4.54e-l	0.31±0.03a-d
Bok Tulsi	3.00±1.29c	23.09±5.95a-e	72.55±4.91 e-m	0.32±0.06a-e
Binna Toa	3.25±0.50cd	24.43±5.10a-g	65.95±2.63b-g	0.37±0.07a-h
Hogla	3.25±0.96cd	21.67±1.48abc	71.57±3.76e-l	0.30±0.08a-d
Kumari	3.50±0.98d	26.76±3.26a-i	68.32±4.54b-h	0.39±0.08b-h
Hijolee	3.50±1.41d	21.50±3.93ab	72.33±5.76e-l	0.30±0.07abc
Marichbati	3.50±1.15d	21.53±2.06ab	75.63±5.03h-n	0.28±0.05a
Sada Aus	3.50±0.82d	24.10±2.55a-f	78.23±6.15j-p	0.31±0.001a-d
Gopal bhog	3.75±1.50de	22.83±4.05a-e	72.20±3.88e-l	0.32±0.06a-f
Aswina	3.75±0.96de	26.12±2.76a-h	66.18±2.75b-g	0.39±0.02b-h
Kataktara	4.25±0.50e	24.68±2.64a-h	74.15±2.05f-m	0.33±0.04a-g
Aus Baku	4.50±1.41e	22.48±1.77a-d	77.13±1.47h-o	0.29±0.001a
Ausa Bogi	4.75±0.96ef	21.83±4.68abc	63.38±3.17b-e	0.34±0.08a-g
Kacha Mota	4.75±1.25ef	23.98±3.23a-f	74.78±6.62g-m	0.32±0.06a-f
Boalia	5.00±1.29ef	23.70±2.64a-f	65.03±1.02b-f	0.36±0.03a-h
Dharial	5.75±1.26f	22.18±3.77abc	65.63±3.15b-g	0.34±0.05a-g
Kumra Gair	6.00±1.29f	22.40±4.23a-d	68.75±3.14b-i	0.33±0.05a-f
SE	0.10	0.36	0.58	0.006
DMRT*	0.29/0.19	1.06/0.71	1.72/1.14	0.02/0.01

[a] accessions in bold letters were also used in experiment-1, SE= Standard error of mean, Mean followed by alphabet showing DMRT ranking: Any two means having a common letter are not significantly different at the 5% level *Largest/smallest shortage significant range values

Appendix: Table 3.8 Cont. Mean and one standard deviation of root dry weight (g) per plant, shoot dry weight (g), total dry weight (g) per plant and root-shoot length ratio of rice accessions in experiment-2

[a]Name of accessions	Drought score	Root dry weight (g)	Shoot dry weight (g)	Root-shoot dry mass ratio	Root dry mass (g) per pot	Shoot dry mass (g) per pot	Total dry weight (g)
Dud Kalam	1.56±0.72a	1.51±0.21	6.33±0.39	0.24±0.05	7.52±1.03	31.62±1.97	7.83±0.30
Aug Meghi	1.63±0.63a	1.43±0.22	6.10±0.58	0.24±0.06	7.13±1.11	30.50±2.91	7.52±0.50
Keora	1.75±0.50a	1.46±0.21	5.78±0.81	0.26±0.05	7.28±1.03	28.89±4.07	7.23±0.73
Manik Mondal	1.75±0.50a	1.23±0.10	5.68±0.51	0.22±0.03	6.16±0.51	28.38±2.52	6.91±0.48
Huma gambir	1.75±0.96a	1.35±0.10	6.20±0.70	0.16±0.10	6.74±0.52	31.00±3.49	7.55±0.69
Agali	1.75±0.50a	1.24±0.06	6.18±1.04	0.21±0.04	6.20±0.31	30.91±5.22	7.42±1.02
Kola Mocha	1.75±0.96a	1.45±0.20	6.20±0.68	0.23±0.03	7.26±1.02	31.00±3.42	7.65±0.82
Dhapa	1.75±0.25a	1.61±0.12	5.98±0.30	0.27±0.02	8.06±0.58	29.90±1.50	7.59±0.37
Bina Muri-2	1.75±0.50a	1.09±0.15	5.05±0.24	0.22±0.03	5.44±0.77	25.25±1.19	6.14±0.35
Nona Balam	1.88±0.48ab	1.83±0.68	7.05±1.66	0.26±0.06	9.14±3.41	35.25±8.31	8.89±2.19
Kala Mona	1.88±0.75ab	1.74±0.14	6.48±0.57	0.27±0.01	8.70±0.68	32.38±2.87	8.22±0.70
Kada Moni	1.90±0.49ab	1.65±0.17	5.53±0.72	0.28±0.01	8.25±0.87	27.62±3.61	7.18±0.80
Bina Muri-1	2.00±0.58ab	1.13±0.20	4.98±0.38	0.23±0.04	5.64±1.01	24.88±1.89	6.11±0.48
Hogla Pata	2.00±0.41ab	1.20±0.08	5.60±0.52	0.22±0.03	6.00±0.41	28.00±2.61	6.80±0.53
Bali Guri	2.12±3.48ab	1.30±0.29	7.02±2.41	0.19±0.05	6.50±1.47	35.12±12.06	8.33±2.58
Aus Nagra	2.25±0.50ab	0.80±0.27	7.08±0.94	0.11±0.02	4.00±1.35	35.37±4.71	7.88±1.17
Hazi Faram	2.25±0.50ab	0.88±0.10	6.70±0.39	0.13±0.02	4.37±0.48	33.50±1.96	7.58±0.35
Tilock Kachari	2.25±0.50ab	1.69±0.28	6.26±0.66	0.27±0.05	8.45±1.39	31.27±3.27	7.95±0.75
Bakee	2.25±0.50ab	0.85±0.06	7.05±0.97	0.12±0.02	4.25±0.29	35.25±4.84	7.90±0.92
Lakhai	2.50±0.82b	1.28±0.05	4.70±0.76	0.28±0.04	6.38±0.25	23.50±3.79	5.98±0.77
Baila Bokri	2.50±1.15b	1.13±0.10	8.55±2.74	0.14±0.03	5.66±0.52	42.75±13.69	9.68±2.82
Kumri Aus	2.50±1.41b	1.03±0.19	9.53±2.97	0.12±0.03	5.12±0.95	47.63±14.85	10.55±2.94
Hasha	2.50±0.96b	1.60±0.54	8.81±2.97	0.16±0.07	7.99±2.71	44.03±14.83	10.40±3.46
Aus Kushi	2.75±0.96bc	1.08±0.21	7.58±1.43	0.14±0.02	5.38±1.03	37.88±7.17	8.65±1.55
Dular	2.75±0.96bc	1.65±0.21	5.98±1.78	0.29±0.07	8.25±1.04	29.90±8.90	7.63±1.93
Hashi Kalmi	2.75±1.26bc	1.48±0.31	5.66±0.92	0.27±0.08	7.38±1.55	28.31±4.59	7.14±0.88
Hanumanjata	2.75±0.96bc	0.90±0.14	5.45±1.27	0.17±0.01	4.50±0.71	27.25±6.38	6.36±1.41
Nuncha	2.75±1.25bc	0.95±0.13	6.35±0.91	0.15±0.01	4.75±0.65	31.75±4.57	7.30±1.03
Panbira-1	2.75±1.25bc	1.15±0.29	6.03±0.33	0.19±0.04	5.75±1.44	30.12±1.65	7.18±0.52
Kartik Sail	3.00±0.58c	0.83±0.25	6.13±0.94	0.14±0.06	4.12±1.1.25	30.62±4.67	6.95±0.72
Panbira-2	3.00±0.58c	1.28±0.29	6.53±1.15	0.20±0.06	6.37±1.44	32.62±5.76	7.80±0.97
Bogi	3.00±0.58c	0.83±0.38	4.80±1.29	0.17±0.04	4.12±1.89	24.00±6.49	5.62±1.64
Bok Tulsi	3.00±1.29c	1.25±0.37	6.15±0.91	0.21±0.06	6.25±1.85	30.75±4.55	7.40±1.00
Binna Toa	3.25±0.50cd	1.57±0.46	6.90±1.06	0.23±0.06	7.88±2.28	34.50±5.30	8.48±1.39
Hogla	3.25±0.96cd	1.08±0.15	5.78±0.65	0.19±0.02	5.36±0.76	28.89±3.26	6.85±0.76
Kumari	3.50±0.98d	1.20±0.21	6.23±0.87	0.19±0.04	5.99±1.04	31.16±4.36	7.43±0.90
Hijolee	3.50±1.41d	1.08±0.35	6.28±0.34	0.17±0.04	5.37±1.75	31.37±1.71	7.36±0.63
Marichbati	3.50±1.15d	1.17±0.21	7.20±1.26	0.16±0.02	5.87±1.03	36.00±6.28	8.38±1.40
Sada Aus	3.50±0.82d	1.10±0.08	8.78±1.51	0.13±0.02	5.50±0.41	43.87±7.57	9.88±1.55
Gopal bhog	3.75±1.50de	0.85±0.10	6.40±0.96	0.13±0.01	4.25±0.50	32.00±4.81	7.25±1.03
Aswina	3.75±0.96de	1.21±0.23	6.90±0.87	0.18±0.03	6.05±1.17	34.51±4.35	8.11±1.02
Kataktara	4.25±0.50e	0.98±0.19	5.98±0.61	0.16±0.02	4.88±0.95	29.88±3.06	6.95±0.79
Aus Baku	4.50±1.41e	1.32±0.41	5.62±0.51	0.23±0.06	6.62±2.06	28.12±2.56	6.95±0.83
Ausa Bogi	4.75±0.96ef	0.90±0.26	4.85±0.61	0.18±0.04	4.50±1.29	24.25±3.07	5.75±0.81
Kacha Mota	4.75±1.25ef	1.33±0.46	6.78±0.33	0.19±0.07	6.62±2.32	33.88±1.65	8.10±0.67
Boalia	5.00±1.29ef	0.98±0.24	7.98±0.33	0.12±0.03	4.88±1.18	39.88±1.65	8.95±0.21
Dharial	5.75±1.26f	1.13±0.46	6.52±1.13	0.17±0.05	5.62±2.29	32.63±5.63	7.64±1.49
Kumra Gair	6.00±1.29f	0.93±0.17	7.15±1.13	0.13±0.02	4.63±0.86	35.75±5.63	8.08±1.18
SE	0.10	0.03	0.11	0.01	0.39	0.59	0.11
DMRT*	0.29/0.01	0.09/0.06	0.33/0.22	0.03/0.02	1.15/0.77	1.75/1.16	0.33/0.22

[a]accessions in bold letters were also used in experiment-1, SE= Standard error of mean, Mean followed by alphabet showing DMRT ranking: Any two means having a common letter are not significantly different at the 5% level, *Largest/smallest shortage significant range values

Appendix: Table 3.9 Mean and one standard deviation of leaf length (cm), leaf width (cm), leaf area (cm2) of rice accessions in experiment-2

[a]Name of accessions	Drought score	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)
Dud Kalam	1.56±0.72a	49.42±3.48e	0.87±0.03a	18.94±2.03d
Aug Meghi	1.63±0.63a	47.93±1.53e	1.35±0.17e	28.19±3.55k
Keora	1.75±0.50a	45.10±2.68c	0.98±0.03b	22.01±1.94ef
Manik Mondal	1.75±0.50a	41.33±3.46b	1.23±0.13d	22.19±4.00f
Huma gambir	1.75±0.96a	45.85±6.84c	1.23±0.19d	24.84±6.65h
Agali	1.75±0.50a	41.78±6.23b	1.13±0.13c	20.55±4.11e
Kola Mocha	1.75±0.96a	51.43±3.26f	1.00±0.00b	22.42±1.42f
Dhapa	1.75±0.25a	43.40±3.49c	1.00±0.10b	18.84±1.77d
Bina Muri-2	1.75±0.50a	42.50±3.90c	1.13±0.05c	20.84±2.08e
Nona Balam	1.88±0.48ab	45.38±4.10c	0.90±0.08a	17.82±2.41c
Kala Mona	1.88±0.75ab	57.98±2.19g	1.08±0.05b	27.19±2.07j
Kada Moni	1.90±0.49ab	44.60±2.38c	1.25±0.13d	24.30±2.68g
Bina Muri-1	2.00±0.58ab	42.93±2.02c	1.20±0.00d	22.45±1.05f
Hogla Pata	2.00±0.41ab	45.25±4.59c	0.90±0.08a	17.87±3.43c
Bali Guri	2.12±3.48ab	36.03±3.26a	1.08±0.05b	16.91±1.98b
Aus Nagra	2.25±0.50ab	50.95±4.90f	1.55±0.06g	34.49±4.27m
Hazi Faram	2.25±0.50ab	52.50±4.00f	1.40±0.00f	32.04±2.44l
Tilock Kachari	2.25±0.50ab	43.50±3.89c	0.85±0.05a	18.13±1.99d
Bakee	2.25±0.50ab	48.18±4.06e	1.30±0.00e	27.30±2.03j
Lakhai	2.50±0.82b	46.40±1.99d	1.10±0.00c	22.25±0.95f
Baila Bokri	2.50±1.15b	48.13±3.89e	1.40±0.08f	29.44±3.64k
Kumri Aus	2.50±1.41b	40.70±2.92b	1.40±0.05f	24.87±2.61h
Hasha	2.50±0.96b	43.68±10.40c	1.18±0.17c	22.86±4.83f
Aus Kushi	2.75±0.96bc	42.83±1.98c	1.25±0.06d	23.34±1.66g
Dular	2.75±0.96bc	40.85±2.41b	1.28± 0.13d	22.77±3.14f
Hashi Kalmi	2.75±1.26bc	36.15±3.75a	1.18±0.05c	18.55±2.39c
Hanumanjata	2.75±0.96bc	44.98±1.24c	1.28±0.05d	25.01±1.43h
Nuncha	2.75±1.25bc	46.95±2.28d	1.03±0.05b	21.00±1.91e
Panbira-1	2.75±1.25bc	45.45±4.01c	1.37±0.19f	27.48±5.85j
Kartik Sail	3.00±0.58c	48.93±3.36e	1.00±0.08b	21.34±2.39e
Panbira-2	3.00±0.58c	46.10±3.07d	0.85±0.05a	18.08±1.70d
Bogi	3.00±0.58c	44.98±2.48c	1.08±0.13b	21.11±2.01e
Bok Tulsi	3.00±1.29c	45.38±3.28c	1.30±0.08e	25.69±2.11h
Binna Toa	3.25±0.50cd	43.00±3.93c	0.98±0.05b	18.28±1.97c
Hogla	3.25±0.96cd	42.04±1.15b	0.92±0.04a	19.98±1.96cd
Kumari	3.50±0.98d	43.53±2.98c	0.81±0.03a	16.76±2.10a
Hijolee	3.50±1.41d	40.18±2.53b	1.20±0.08d	21.04±2.20e
Marichbati	3.50±1.15d	45.43±2.00c	1.23±0.05d	24.29±2.03g
Sada Aus	3.50±0.82d	49.75±7.46f	1.25±0.17d	27.51±7.73j
Gopal bhog	3.75±1.50de	48.12±2.60e	1.25±0.08d	26.14±1.63I
Aswina	3.75±0.96de	40.18±2.89b	0.87±0.04a	15.16±0.99a
Kataktara	4.25±0.50e	45.68±2.30c	1.38±0.05f	27.41±2.16j
Aus Baku	4.50±1.41e	44.25±2.36c	1.38±0.10f	26.51±2.10I
Ausa Bogi	4.75±0.96ef	44.83±2.78c	1.23±0.17d	23.87±3.05g
Kacha Mota	4.75±1.25ef	50.08±2.11f	0.88±0.05a	19.12±1.69d
Boalia	5.00±1.29ef	42.63±1.78c	1.23±0.05d	22.75±1.28f
Dharial	5.75±1.26f	39.15±1.84b	1.20±0.00d	20.48±0.97e
Kumra Gair	6.00±1.29f	48.23±3.63e	1.00±0.00b	21.03±1.58e
SE	0.10	0.38	0.02	0.48
DMRT*	0.29/0.19	1.13/0.75	0.06/0.04	1.43/0.94

[a]accessions in bold letters were also used in experiment-1, SE= Standard error of mean, Mean followed by alphabet showing DMRT ranking: Any two means having a common letter are not significantly different at the 5% level, *Largest/smallest shortage significant range values

Appendix: Table 3.9 Cont. stomata number (per mm^2), stomata conductance (cm/s) before and at stress conditions of rice accessions in experiment-2

[a]Name of accessions	Drought score	Leaf stomata number	Conductance (cm/s) before	Conductance (cm/s) at stress
Dud Kalam	1.56±0.72a	130±29abc	0.43±0.05Ii	0.06±0.007a
Aug Meghi	1.63±0.63a	115±29ab	0.38±0.02d-i	0.07±0.02b
Keora	1.75±0.50a	116±28ab	0.35±0.03c-i	0.06±0.005a
Manik Mondal	1.75±0.50a	115±6ab	0.37±0.03d-i	0.07±0.02b
Huma gambir	1.75±0.96a	152±21b-e	0.37±0.02d-i	0.07±0.004b
Agali	1.75±0.50a	134±37a-d	0.38±0.13e-i	0.06±0.006a
Kola Mocha	1.75±0.96a	110±10a	0.38±0.04d-i	0.07±0.01b
Dhapa	1.75±0.25a	120±22abc	0.37±0.03d-i	0.06±0.006a
Bina Muri-2	1.75±0.50a	120±25abc	0.37±0.10d-i	0.07±0.008b
Nona Balam	1.88±0.48ab	122±25abc	0.37±0.02d-i	0.08±0.01c
Kala Mona	1.88±0.75ab	108±12a	0.38±0.02d-i	0.07±0.01b
Kada Moni	1.90±0.49ab	127±30abc	0.40±0.04f-i	0.06±0.004a
Bina Muri-1	2.00±0.58ab	132±37a-d	0.38±0.05d-i	0.06±0.007a
Hogla Pata	2.00±0.41ab	116±28ab	0.39±0.01f-i	0.08±0.02c
Bali Guri	2.12±3.48ab	158±8cde	0.41±0.08hi	0.08±0.01c
Aus Nagra	2.25±0.50ab	142±12a-e	0.30±0.05a-i	0.09±0.01d
Hazi Faram	2.25±0.50ab	110±17a	0.23±0.06abc	0.07±0.008b
Tilock Kachari	2.25±0.50ab	116±9ab	0.37±0.02d-i	0.07±0.02b
Bakee	2.25±0.50ab	145±31a-e	0.32±0.05b-i	0.08±0.01c
Lakhai	2.50±0.82b	143±22a-e	0.29±0.07a-h	0.08±0.01c
Baila Bokri	2.50±1.15b	113±7ab	0.31±0.07b-i	0.08±0.02c
Kumri Aus	2.50±1.41b	126±13abc	0.32±0.03b-i	0.08±0.01c
Hasha	2.50±0.96b	133±29a-d	0.36±0.08c-i	0.09±0.02d
Aus Kushi	2.75±0.96bc	146±26a-e	0.35±0.10c-i	0.09±0.02d
Dular	2.75±0.96bc	135±26a-d	0.39±0.06e-i	0.10±0.01e
Hashi Kalmi	2.75±1.26bc	119±13abc	0.41±0.0ghi	0.07±0.02b
Hanumanjata	2.75±0.96bc	152±22b-e	0.35±0.07c-i	0.08±0.01c
Nuncha	2.75±1.25bc	159±36cde	0.30±0.12a-i	0.08±0.02c
Panbira-1	2.75±1.25bc	132±24a-d	0.38±0.16d-i	0.10±0.01e
Kartik Sail	3.00±0.58c	137±20a-e	0.31±0.14a-i	0.09±0.01d
Panbira-2	3.00±0.58c	144±13a-e	0.26±0.11a-e	0.09±0.02d
Bogi	3.00±0.58c	146±20a-e	0.34±0.12c-i	0.09±0.01d
Bok Tulsi	3.00±1.29c	142±28a-e	0.30±0.06a-i	0.07±0.01b
Binna Toa	3.25±0.50cd	134±16a-d	0.26±0.06a-f	0.07±0.007b
Hogla	3.25±0.96cd	123±8abc	0.27±0.02a-i	0.09±0.01d
Kumari	3.50±0.98d	127±8abc	0.30±0.03a-i	0.09±0.009d
Hijolee	3.50±1.41d	142±37a-e	0.32±0.12c-I	0.08±0.01c
Marichbati	3.50±1.15d	122±16abc	0.33±0.11c-i	0.07±0.02b
Sada Aus	3.50±0.82d	117±4ab	0.19±0.04ab	0.09±0.008d
Gopal bhog	3.75±1.50de	145±15a-e	0.36±0.09c-i	0.10±0.01e
Aswina	3.75±0.96de	111±15a	0.24±0.02b-d	0.09±0.02d
Kataktara	4.25±0.50e	125±15abc	0.25±0.02a-d	0.09±0.01d
Aus Baku	4.50±1.41e	169±19de	0.37±0.09d-i	0.07±0.01b
Ausa Bogi	4.75±0.96ef	116±16ab	0.34±0.10c-i	0.09±0.03d
Kacha Mota	4.75±1.25ef	175±11e	0.35±0.10c-i	0.095±0.01d
Boalia	5.00±1.29ef	122±9abc	0.26±0.08a-f	0.07±0.01d
Dharial	5.75±1.26f	138±18a-e	0.29±0.09a-h	0.08±0.01c
Kumra Gair	6.00±1.29f	114±28ab	0.18±0.04a	0.10±0.01e
SE	0.10	2.00	0.006	0.001
DMRT*	0.29/0.19	5.94/3.93	0.02/0.01	0.002/0.001

[a]accessions in bold letters were also used in experiment-1, SE= Standard error of mean, Mean followed by alphabet showing DMRT ranking: Any two means having a common letter are not significantly different at the 5% level, *Largest/smallest shortage significant range values

Appendix: Table 3.10 Different principal components and their variance (%) extracted by principal component analysis on the basis of physio-morphological characteristics in experiment-2

Principal Components	Initial Eigen values			Extraction sums of squared loadings		
	Total	% of variance	Cumulative %	Total	% of Variance	Cumulative %
1	6.05	31.81	31.81	6.05	31.81	31.81
2	4.68	24.64	56.45	4.68	24.64	56.45
3	2.67	14.06	70.52	2.67	14.06	70.52
4	1.14	5.99	76.50	1.14	5.99	76.50
5	1.11	5.86	82.27	1.11	5.86	82.37
6	0.65	3.41	85.78			
7	0.59	3.09	88.87			
8	0.50	2.61	91.48			
9	0.45	2.24	93.82			
10	0.39	2.06	95.88			

Appendix: Table 3.11 Stomata conductance reduction (%) and root contribute to total biomass (%) of rice accessions in experiment-1

Name of Accessions	Drought score	Conductance reduction (%)	% of root contribute to total biomass
Aswina	2.63	25.79	20.62
Dud Kalam	1.75	12.16	15.99
Dhapa	1.63	12.63	23.09
Dud Mona	3.25	15.92	15.51
Hogla	1.63	32.16	17.40
Kumari	2.38	19.88	15.49
Hogla Pata	3.75	20.86	18.30
Kada Moni	3.00	11.22	30.26
Kajal Sail	3.50	23.86	14.65
Kala Mona	1.75	15.89	25.57
Kumra Gair	7.88	37.25	15.99
Kacha Mota	5.00	31.43	15.95
Kartik Sail	2.75	23.30	20.73
Kola Mocha	3.63	28.05	17.20
Lakhai	5.25	21.08	18.57
Nuncha	4.63	28.66	14.47
Nona Balam	4.88	22.86	21.55
Panbira-2	4.63	26.45	13.02
Raja Sail	2.88	29.69	18.45
Tilock Kachari	1.69	15.62	23.45
Keora	1.56	12.38	29.73

Appendix: Table 3.12 Regression coefficients of water use efficiency (g/kg) (dependent variable) to independent variables of root dry weight (g), shoot dry weight (g), root-shoot dry weight ratio, root-shoot length ratio, stomata conductance (cm/s) and evapotranspiration (mm/day) in experiment-2

Parameters	Un-standardized coefficients		Standardized coefficients	t	Sig.
	B	Std Error	Beta		
Constant	4.671	0.39		11.936	0.89
Stomata conductance (cm/s) before stress	0.86	0.19	0.059	4.38	0.000
Total dry mass (g) per plant	0.85	0.012	1.033	73.73	0.000
ET (mm/day)	-1.47	0.14	-0.152	-10.54	0.000

Sig. = Significant level, t= student test

Appendix: Table 3.12a Regression model for water use efficiency in experiment-2

Model	R	R^2	Adjusted R^2	Est. std error
1	0.98	0.97	0.97	0.23

Appendix: Table 3.12b Analysis of variance for regression model of dependent variable water use efficiency in experiment-2

Source of variation	Sum of square	df	Mean square	F	Sig.
Regression	298.46	3	97.488	1943.88	0.000
Residual	9.62	188	0.051		
Total	308.086	191			

D.f.= Degree of freedom

Appendix: Table 3.13 Regression coefficients of water use efficiency (dependent variable) to independent variables of root length (cm), shoot length (cm), root dry weight (g), shoot dry weight (g), dry weight ratio, root-shoot length ratio, stomata conductance (cm/s) and evapotranspiration (mm/day) in combination of two experiments

Parameters	Un-standardized coefficients		Standardized coefficients	t	Sig.
	B	Std Error	Beta		
Constant	0.11	0.217		0.548	0.58
Total drymass (g) per plant	0.403	0.008	0.97	52.52	0.000
ET (mm/day)	-0.11	0.06	-0.03	-1.83	0.05

Sig. = Significant level, t= student test

Appendix: Table 3.13a Regression model for dependent variable water use efficiency in combination of two experiments

Model	R	R^2	Adjusted R^2	Est. std error
	0.99	0.99	0.99	0.118

Appendix: Table 3.13b Analysis of variance for regression model of dependent variable water use efficiency in combination of two experiments

Source of variation	Sum of square	Df	Mean square	F	Sig.
Regression	91.78	2	45.663	3269.81	0.000
Residual	0.92	66	0.014		
Total	92.25	68			

D.f.= Degree of freedom

Appendix: Table 4.1 Different principal components and their variance (%) extracted by principal component analysis based on isozyme data

Principal Components	Initial Eigen values			Extraction sums of squared loadings		
	Total	% of variance	Cumulative %	Total	% of Variance	Cumulative %
1	12.06	12.44	12.44	12.06	12.44	12.44
2	9.72	10.02	22.46	9.72	10.02	22.46
3	7.95	8.19	30.65	7.95	8.19	30.65
4	7.45	7.68	38.33	7.45	7.68	38.33
5	6.33	6.53	44.85	6.33	6.53	44.85
6	5.74	5.92	50.77	5.74	5.92	50.77
7	4.97	5.12	55.89	4.97	5.12	55.89
8	4.43	4.57	60.46	4.43	4.57	60.46
9	4.05	4.17	64.63	4.05	4.17	64.63
10	3.61	3.72	68.35	3.61	3.72	68.35
11	3.32	3.42	71.77	3.32	3.42	71.77
12	2.99	3.08	74.85	2.99	3.08	74.85
13	2.71	2.79	77.65	2.71	2.79	77.65
14	2.65	2.73	80.37	2.65	2.73	80.37
15	2.20	2.27	82.65	2.20	2.27	82.65
16	2.00	2.06	84.70	2.00	2.06	84.70
17	1.80	1.86	86.56	1.80	1.86	86.56
18	1.63	1.68	88.25	1.63	1.68	88.25
19	1.54	1.59	89.83	1.54	1.59	89.83
20	1.39	1.43	91.26	1.39	1.43	91.26
21	1.28	1.32	92.58	1.28	1.32	92.58
22	1.05	1.08	93.66	1.05	1.08	93.66

Appendix: Table 5.1 Effect of boric acid on pollen burst and pollen germination of rice

	0.001	0.002	0.003	0.005	0.007	0.008	0.009	0.01	0.02
No. Pollen Grain	279.8± 47.4	313.7± 47.3	308.7± 51.8	341. 7±45.4	333.7± 42.4	294.3± 21.4	298.3± 45.8	325.7± 26.6	283.7± 37.1
Pollen Burst	163.7 ± 26.1 (58.5)	114.1 ± 22.1 (49.1)	219 ± (61.4)	126 ± (57.8)	138 ± (55.0)	175± (61.8)	161± (58.3)	104± (48.2)	157± (48.5)
Germination	0	0	0	0	0	0	0	0	0

0.02% $CaCl_2$, 0.01% Potassium phosphate, 10% Sucrose; Figure in parenthesis indicate the percentage

Appendix: Table 5.2 Effect of boric acid on pollen germination of rice at a calcium chloride concentration of 0.03%

	0.001	0.002	0.003	0.005	0.007	0.008	0.009	0.01	0.02
No. Pollen Grain	276	341	311	269	326	293	256	238	279
Pollen Burst	191 (69.21)	216 (63.34)	234 (75.24)	185 (68.77)	247 (75.77)	198 (67.58)	187 (73.05)	147 (61.76)	158 (56.63)
Germination	0%	0%	0%	0%	0%	0%	0%	0%	0%

0.03% $CaCl_2$, 0.01% Potassium phosphate, 15% Sucrose, Figure in parenthesis indicate the percentage

Appendix: Table 5.3 Effect of boric acid on pollen germination of rice at a calcium chloride concentration of 0.04%

	0.001	0.002	0.003	0.005	0.007	0.008	0.009	0.01	0.02
No Pollen Grain	326	253	274	326	285	224	267	241	302
Pollen Burst	195 (59.8)	162 (64.0)	198 (72.3)	247 (75.8)	209 (73.3)	183 (81.7)	189 (71.3)	137 (56.9)	181 (59.9)
Germination									

0.04% $CaCl_2$, 0.01% Potassium phosphate, 20% Sucrose; Figure in parenthesis indicate the percentage

Appendix: Table 5.4 Effect of calcium nitrate on pollen germination of rice

Con.	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.1	0.5	1%
No. Pollen Grain	246	283	268	403	259	276	318	176	271	227
Pollen Burst	85 (34.6)	94 (33.2)	96 (35.8)	140 (34.7)	105 (40.5)	91 (32.9)	127 (39.9)	89 (50.6)	152 (56.1)	102 (44.9)
Germi nation										

0.008% Boric acid, 0.01% Potassium phosphate, 0.01% Magnesium sulphate and 10% Sucrose; Figure in parenthesis indicate the percentage

Appendix: Table 5.5 Effect of calcium chloride on pollen germination of rice at a boric acid concentration of 0.008%

	0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.1	0.5	1%
No Pollen Grain	254	211	312	297	263	241	285	316	239	278	245
Pollen Burst	87 (34.3)	137 (64.9)	193 (61.9)	201 (67.7)	185 (70.3)	203 (84.2)	194 (68.1)	206 (65.2)	143 (59.8)	132 (55.7)	108 (44.1)
Germi nation											

0.008% Boric acid, 0.01% Potassium phosphate, 0.01% Magnesium sulphate and 15% Sucrose; Figure in parenthesis showing the percentage

Appendix: Table 5.6 Effect of calcium chloride on pollen germination of rice at a boric acid concentration of 0.01%

	0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.1	0.5	1%
No Pollen Grain	244	308	275	269	251	321	258	236	285	257	274
Pollen Burst	128 (52.5)	152 (49.4)	135 (49.1)	125 (46.5)	149 (59.4)	199 (62.0)	155 (60.1)	133 (56.4)	163 (57.2)	138 (53.7)	151 (55.1)
Germi nation											

0.01% Boric acid, 0.01% Potassium phosphate, 0.01% Magnesium sulphate and 15% Sucrose; Figure in parenthesis indicates the percentage

Appendix: Table 5.7 Effect of calcium chloride on pollen germination of rice at a boric acid concentration of 0.02%

	0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.1	0.5	1%
No Pollen Grain	214 ±	298	189	319	347	238	263	236	285	257	274
Pollen Burst	105 (49.1)	154 (51.7)	115 (60.9)	163 (51.1)	216 (62.3)	171 (71.9)	171 (65.0)	133 (56.4)	163 (57.2)	138 (53.7)	151 (55.1)
Germination											

0.02% Boric acid, 0.01% Potassium phosphate, 0.01% Magnesium sulphate and 15% Sucrose; Figure in parenthesis indicate the percentage

Appendix: Table 5.8 Effect of Potassium phosphate on rice pollen germination

Concent.	0.0	0.01	0.02	0.03	0.04	0.05	0.1	0.5
No Pollen Grain	218	234	316	283	254	260	296	331
Pollen Burst	134 (61.47)	203 (86.75)	225 (71.20)	184 (65.02)	161 (63.39)	112 (43.08)	161 (54.39)	129 (38.97)
Germination	0	0	0	00	0	0	0	0

0.02% Boric acid, 0.01% Potassium phosphate, 0.01% Magnesium sulphate and 15% Sucrose; Figure in parenthesis indicate the percentage