MOLECULAR AND PHYSIOLOGICAL ANALYSIS OF TRANSGENIC RICE HARBOURING CHIMERIC *PDH47* GENE AGAINST ABIOTIC STRESS TOLERANCE

A Thesis

Submitted to the

Assam Agricultural University

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY (Agriculture)

IN

AGRICULTURAL BIOTECHNOLOGY

By

Dimple Boro

Registration No. 579 of 2014

[Roll No. 2014-ADJ-04]

DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

ASSAM AGRICULTURAL UNIVERSITY

JORHAT-785013 (ASSAM)

February, 2020

ASSAM AGRICULTURAL UNIVERSITY
FACULTY OF AGRICULTURE

CERTIFICATE - I

This is to certify that the thesis entitled **"Molecular and physiological analysis of transgenic rice harbouring chimeric *PDH47* gene against abiotic stress tolerance"** submitted to the Faculty of Agriculture, Assam Agricultural University in partial fulfillment of the degree of **Doctor of Philosophy (Agriculture)** in **Agricultural Biotechnology** is a record of research work carried out by **Dimple Boro** under my personal supervision and guidance.

All help received by her have been duly acknowledged.

No part of this thesis has been reproduced elsewhere for any degree.

Dated: Jorhat
The ……. 2020

_____
(Salvinder Singh)
Major Advisor
Professor
Department of Agricultural Biotechnology
College of Agriculture
AAU, Jorhat-13

CERTIFICATE - II

This is to certify that the thesis entitled **"Molecular and physiological analysis of transgenic rice harbouring chimeric *PDH47* gene against abiotic stress tolerance"** submitted by **Dimple Boro, Roll No. 2014-ADJ-04** to the Assam Agricultural University in partial fulfillment of the requirement for the degree of **Doctor of Philosophy (Agriculture)** in the discipline of **Agricultural Biotechnology** has been examined and approved by the Student's Advisory Committee and the External Examiner, after viva-voce.

_____ _____

(Salvinder Singh) External examiner
Major Adviser & Chairman

Members of the Advisory Committee:

1. _____
   (M. K. Modi)

2. _____
   (P. Kalita)

3. _____
   (A.M. Baruah)

_____ _____

(M.K. Modi)
Professor & Head
Department of Agril. Biotechnology
Assam Agricultural University
Jorhat-785 013 (Assam)

(A. Baishya)
Director
Post Graduate Studies
Assam Agricultural University
Jorhat-785 013 (Assam)

ACKNOWLEDGEMENT

It is a pleasure to express her regards and thanks to all of those, who helped her towards the completion of this thesis.

She wishes to express her deep sense of gratitude and regards to her Major Advisor and Chairman of the Advisory Committee, Dr. S. Singh, Professor, Department of Agricultural Biotechnology, Faculty of Agriculture, Assam Agricultural University, Jorhat, for his initial support and valuable guidance. His patience and insight into the subject has always made her understand it in a broader way and even his motivational suggestions to help her realize in preparing the strategy for the research work and in writing the manuscript.

She also extends her heartfelt thanks to the members of the advisory committee, Dr. M.K. Modi, Professor and Head of the Department of Agricultural Biotechnology, AAU, Jorhat-13, Assam, Dr. P. Kalita, Professor, Department of Crop Physiology, AAU, Jorhat-13, Assam and Dr. A.M. Baruah, Professor, Department of Biochemistry and Agricultural Chemistry, AAU, Jorhat-13, Assam for their immense help and support during the course of investigation. She also would like to express her thanks to Dr. B.K. Sarmah, ICAR-National Professor and Director, Department of Agricultural Biotechnology, AAU, Jorhat for allowing her to do her Research works in the DBT-AAU Centre, AAU, Jorhat-13, Assam.

The authoress also wishes to put forward her heartiest thanks to Dr. K.M. Bujarbaruah, Former Hon'ble Vice-Chancellor, AAU, Jorhat, Dr. A. Baishya, Director of Post-Graduate Studies and Dr. Madhumita Borooah, Professor, Department of Agricultural Biotechnology, Dr. Aishawarya Baruah, Assistant Professor, Department of Agricultural Biotechnology and Dr. A.R. Baruah, Assistant Professor, Department of Agricultural Biotechnology, AAU, Jorhat for their valuable suggestions during the phase of the research work.

She would like to express special thanks to Dr. Dhanwantari L. Singha for her valuable suggestion and constant support at all times.

She also wishes to acknowledge Ministry of Tribal Affairs, Government of India, for selecting her in the merit list category all over India by providing her the NFHE fellowship per year to precede her Ph.D research work. She gratefully acknowledges the Department of Agricultural Biotechnology, DBT-AAU Centre, Distributed Information Centre, Department of Soil Science, Department of Agricultural Engineering, Assam Agricultural University, Jorhat for providing necessary facilities to undertake this research work.

She would like to express her thanks to all the faculty members of the Department of Agricultural Biotechnology for their encourgament, support and guidance throughout the entire period of the research work.

She would like to thank Dr.Pradeep Patel, Scientist, Department of Plant Physiology and Breeding, Tocklai Tea Research Institute for helping her to have a chance to use the IRGA instrument in his department. Moreover, she would like to express her heartiest thanks to Sarbani Roy, Technical Officer, Tocklai Research Institute, for all her unconditional support to help her having a chance to do her one of the physiological work in their department.

She also like to thank to Dr. Sangeeta Sharma, Dr. Dhananjay Sharma, Dr. Wasifur Rahman, Dr. Trishna Konwar, Mamta Bhattacharjee, Dr. Mahesh Jagadale, Rouf Ahmed, Birla, Dr. Richa Sharma, Sangeeta Bhandari, Abhik Limasunep Longkumer, Dr. Gajendra Mohan Baldodiya, Jyotsna Dayma, Ricky, Ruby Gupta, Sudipta Bhattacharyya, Gracia, Swapnil Kumar Meshram, Kamalakshi Saikia, Bharati Deori and Ajay Keot and all her batchmates and labmates for their kind help and support.

She also thanks to Dr. Debajit Das who always inspired her to work with more enthusiasm towards achieving her aims and her warm thanks to Dr. Trans Ncog Hee who have helped her in some aspects and provided his assistance.

She also thankful to Jun Da, Bhabesh Da, Rupam Da, Geetu Ba, Ajay Da, Mridul, Sarika Ba, Manash, Bipul Da and all the official staffs of the Department of Agricultural Biotechnology for all their help and co-operation rendered to her.

She also wishes to express her sincere thanks to her parents, her grandmom and her younger brother Dr. Manjit Boro and her best friends Sabita Sharma and Taposi Kalita for their immense love, blessings, sacrifices, guidance, love, care, affection, sincere advice,motivations and invaluable emotional support bestowed on her and for the constant encouragement which made her to achieve her goal.

Last but not the least, she also thankful to Almighty for giving her the insight in choosing her path and the courage in following it.

Dated: Jorhat

The..... 2020 *The authoress*

ABSTRACT

Abiotic stress especially drought can severely affect the physiological status of any plants; thereby impart significant negative impact on growth, development, and metabolism. The major emphasis of most of the recent studies was identification of stress-regulated genes and transcription factors which play important role in governing tolerance/resistance against abiotic stresses. DEAD-box helicases (Asp-Glu-Ala-Asp amino acid) are one of the important genes which confer tolerance to various abiotic stresses. They are involved in unwinding of nucleic acids by utilizing the energy from ATP hydrolysis. *PDH47* (Pea DNA Helicase 47kDa) gene, one of the DEAD-box helicases is known to impart various abiotic stress tolerance. In the present study rice transgenic line cv. IR64 was developed through *Agrobacterium* mediated genetic transformation using immature embryos as explants. The putative transgenic lines showed presence of transgene when subjected to PCR analysis using gene specific primers. Three previously developed transgenic rice lines in our laboratory namely ASD16-46/1, ASD16-66/1 and ASD16-68/1 expressing *PDH47* gene were selected for drought stress tolerance study. Quantitative Real Time PCR analysis showed varied level of expression of *PDH47* gene both in the root and leaf tissues of transgenic line before and after drought stress. The expression of *PDH47* gene induced during drought stress in the transgenic lines, showed varied level of drought tolerance in the vegetative stage without any negative effects on the morphological and agronomical traits. The physiological and biochemical analyses confirmed that the expression of *PDH47* gene in the transgenic lines was associated with increased leaf relative water content, water retention capacity, maintenance of chlorophyll, stomatal conductance, net photosynthetic rate, transpiration rate and water use efficiency. These transgenic lines also showed an increased accumulation of the osmolytes like proline, glycine betaine and decreased electrolyte leakage, lipid peroxidation, less accumulation of H_2O_2 during drought stress. These transgenic lines showed better root architecture system such as root length, root number, root thickness, root biomass, shoot biomass and root to shoot ratio as compared to control untransformed plants during drought stress. The western blot analysis confirmed that the expression of RUBISCO large subunit (55kDa) protein was more in the transgenic rice lines as compared to untransformed control plants during drought stress treatment.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABBREVIATIONS AND SYMBOLS USED

$	: US Dollars
%	: Per cent
°C	: Degree Centigrade
µg	: microgram
µl	: microlitre
µM	: micromolar
2, 4-D	: 2, 4- dicholorophenoxyacetic acid
ABA	: Abscisic acid
ATP	: Adenosine triphosphate
BAP	: 6- benzlaminopurine
bp	: Basepair
CaMV	: Cauliflower mosaic virus
CCM	: Cell Culture medium
cDNA	: Complementary DNA
C_c	: CO_2 concentration
CFU	: Colony forming unit
cm	: Centimeter
CO_2	: Carbon dioxide
cv	: Cultivar
d	: day(s)
DAB	: 3, 3'-diaminobenzidine
DAP	: Days after pollination
df	: degree of freedom
DEPC	: Diethyl pyrocarbonate
DNA	: Deoxyribonucleic acid
DNase	: Deoxyribonuclease
dNTP	: Deoxynucleoside triphosphate
dscDNA	: Double stranded complementary DNA
e.g.	: Exempli gratia (for example)
EDTA	: Ethylene diaminetera acetic acid
EL	: Electrolyte Leakage
EST	: Expressed Sequence Tag

et. al.	: Etalia
g	: gramme(s)
GB	: Glycine Betaine
hr	: hour(s)
H_2O_2	: Hydrogen peroxide
hpt	: hygromycin phospotransferase gene
i.e.	: Id est (That is)
IRGA	: Infrared Gas Analyzer
kbp	: kilo basepair
kg	: kilogramme(s)
l	: litre
LB	: Luria –Bertani
LP	: Lipid Peroxidation
mRNA	: Messenger RNA
M	: molar
MDA	: Malondialdehyde
MCS	: Multiple Cloning Site
MW	: Molecular weight
mg	: Milli gram
mg/L	: Milli gram per Litre
min	: Minutes
ml	: Milli litre
mM	: milimolar
MS	: Murashige and Skoog
MW	: Molecular weight
N	: Normality
NAA	: α –napthaleneacetic acid
ng	: nanogram
OD	: Optical Density
PCR	: Polymerase chain reaction
pH	: Potential of hydrogen
PSII	: Photosystem II
qPCR	: Quantitative Polymerase Chain Reaction
RbcL	: Rubisco Large Subunit

RH	: Relative Humidity
RNA	: Ribonucleic acid
ROS	: Reactive oxygen species
rpm	: Revolution per minute
RSA	: Root System Architecture
RT- PCR	: Reverse Transcription Polymerase chain Reaction
RWC	: Relative Water Content
s	: second(s)
SDS	: Sodium dodecyl sulphate
TAE	: Tris-acetate EDTA
TBA	: Thiobarbituric Acid
TBE	: Tris-borate EDTA
T-DNA	: Transferred-DNA
TE	: Tris-EDTA
U	: Unit (s)
V	: volts
Viz	: Videlicet
w/v	: weight/volume
wk	: week(s)
WRC	: Water Retention Capacity
WUE	: Water Use efficiency
χ^2	: Chi-square

Introduction…✍

CHAPTER I

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important staple food crops in the world. Almost half of the world's population depend on rice as their staple food (Coffman and Juliano, 1987). Rice provides 21% of global human per capita energy and 15% of per capita protein (International Rice Research Institute, 2013). Among the South Asian countries, rice holds special attention in the Indian economy. India is the largest exporter and the fourth largest rice-consuming nation in the South Asian region (Ghose *et al*., 2013). The major rice growing states in India are West Bengal, Uttar Pradesh, Andhra Pradesh, Punjab, Bihar, Orissa, Chattisgarh, Assam, Tamil Nadu and Haryana. In north-eastern part of the country rice occupies special position among the other cereal crops, occupying about 3.51 million hectares accounting for more than 80% of the total cultivated area (Ngachan *et al.,* 2011). Rice is mainly a kharif crop in India which needs temperature of around 25° C and requires a heavy annual rainfall of more than 100 cm. In Assam, rice is grown during summer, autumn and winter seasons. However, the existing rice varieties cannot yield to its maximum potential. One of the major reasons behind the threats to the sustainable rice production is prevalence of various abiotic stresses such as drought, salinity, high temperature *etc*. in different stages of growth (Chinnusamy *et al.,* 2003). According to Widawsky and O'Toole (1990) and Evenson *et al*., (1996) drought alone can reduce the rice production of Eastern India to an extent of 2.9 million metric tons annually whereas, crops with abiotic stress resistance traits can adapt well in substandard growth conditions and thus contribute to increase in overall food production (Chen *et al*., 2012). Prevalence of drought depends upon many factors such as occurrence and distribution pattern of rainfall, evaporative demands and moisture storing capacity of soils. Though the occurrence of drought in Assam is rare, it is reported that it occurs once in every 15 year (http://www.nih.ernet.in/rbis/India_Information/draught.htm). Assam has witnessed unprecedented droughts during 2005-2006 and subsequently in 2009-2011 which caused heavy damage to cereal crops including rice (Parida and Oinam, 2015). Therefore, it has become imperative to develop sustainable drought tolerant/resistant rice varieties in order to mitigate the annual yield loss.

Rice production should increase by at least 60% during 2050 from the current level of production to meets the increasing demands. Abiotic stress particularly drought pose serious threat to rice production. Conventional breeding has met with limited success due to complexity of drought tolerance mechanisms and also due to limited genetic variation for drought tolerance in rice germplasm. In this context, transgenic approach enables us to empower rice to withstand drought by incorporating stress responsive genes from other sources. Identification and transfer of drought tolerance genes through transgenic research is one of the promising solutions for protecting crops against drought stress.

Through the course of evolution, plants have developed sophisticated defense mechanisms to withstand different environmental stresses both biotic and abiotic by various biochemical and physiological changes that results in the expression of many stress responsive genes (Datta *et al*., 2012). For designing a strategy to enhance the survivability of plants in adverse environmental conditions, biotechnological approaches have proved to be a successful one. In the last two decades, significant advances have been made in the areas of rice molecular biology and biotechnology and transgenic approach is found to be suitable one to address the problem of annual yield loss of rice due to occurrence of a number of abiotic stresses during its growing period. A number of genes have been introduced in the cultivated rice germplasm using the transgenic technology in order to develop abiotic stress tolerant/resistant cultivar which includes overproduction of enzymes responsible for biosynthesis of osmolytes, late-embryogenesis abundant (LEA) proteins and detoxifying enzymes that are responsible for reducing the overproduction of reactive oxygen species (ROS) (Bohnert and Jensen, 1996). Varshney *et al.,* (2011) reported that the introgession of genes encoding several transcription factors results in expression of several downstream genes responsible for dehydration stress tolerance.

It is well reported that many genes of the nucleic acid pathways (including helicases) are highly up-regulated in response to both cold and salinity stresses indicating their involvement in stress signalling (Boyer, 1982; Khush, 1999; Jones and Jones, 1989). Helicases are ubiquitous molecular motor protein known for its involvement in plant growth and development in an ATP-dependent manner by playing an important role in maintaining the genome integrity in both prokaryotes and eukaryotes and thus participates in such abiotic stress responses (Vashisht and Tuteja, 2005). The role of DNA helicases is to unwind duplex DNA and thus involved in

replication, repair, recombination and transcription, whereas RNA helicases unfold the secondary structures in RNA and thereby involved in transcription, ribosome biogenesis and translation initiation (Matson, 1991; Tuteja, 2003; Luking *et al.,* 1998). The effect of drought stress on plants may range from morphological to molecular changes and can be seen in any physiological stages of plant growth and development (Farooq *et al.,* 2008). Drought stress affect severely in two of the critical stages of rice plants *i.e.* vegetative growth and flowering stage, due to which the yield of the crop reduces to significant extent (Yang *et al*., 2019). The morphological effects include reduction in leaf size, stems extension, root proliferation and all these effects cumulatively disturbs the plant water relations by closing stomata and thereby reducing the water use efficiency (WUE) (Murtaza *et al*., 2016). It is well documented that some class of helicases significantly up-regulated during abiotic stresses (Nidumukkala *et al*., 2019). D-E-A-D box helicases are one of them, which are members of protein family that has one of the conserved D-E-A-D/H or D-E-X-H regions (Asp-Glu-Ala-Asp/His, X can be any amino acid) (Tuteja *et al.* 2012). DEAD box helicase protein family contains two domains-an N-terminal domain and a C-terminal domain with nine highly conserved motifs (Q, I, Ia, Ib, II, III, IV, V and VI) within these domains (Tuteja *et al.*, 2012). N-terminal domain consists of 6 motifs (Q, I, Ia, Ib, II and III) whereas C-terminal domain consists of 3 motifs (IV, V and VI). These conserve regions are important for the helicase function of the protein. The proteins of DEAD-box family are found in eukaryotes as well as in prokaryotes (Aubourg *et al.,* 1999; de la Cruz *et al.,* 1999; Rocak and Linder, 2004).

Several studies suggested that the DEAD-box protein plays important role in plant growth and development in response to biotic and abiotic stresses. It has been demonstrated that over-expression of DEAD-box helicase (*PDH45*) in rice confers tolerance to salinity at seedling and reproductive stages (Amin *et al*., 2012). Rice DEAD box protein, *OsABP* was reported to be up-regulated in response to multiple abiotic stresses including salt, dehydration, ABA, blue and red light (Macovei *et al.*, 2012). A putative DEAD box RNA helicase gene, *SIDEAD31* from tomato, regulates salt and drought tolerance in tomato (Zhu *et al.*, 2015). The pea DNA helicase 47 (*PDH47*) is one such important gene of DEAD box family having multiple roles. The transcript of *PDH47* gene of *Pisum sativum* was induced under cold (4˚C) and salt stress (Vashisht and Tuteja, 2005; Vashisht *et al.*, 2005) indicating its involvement in the cold signalling pathways. Moreover, Singha *et al*., (2017) reported that the heterologous expression of

PDH47 gene confers drought tolerance in *indica* rice variety. DEAD–box helicase genes were reported to be induced under conditions of chilling and freezing stress (Chamot *et al.*, 1999; Gong *et al.*, 2002).

The Plant Transformation laboratory, Department of Agricultural Biotechnology, Assam Agricultural University, Jorhat had developed several transgenic *indica* rice cultivars like IR64 and ASD16 harbouring chimeric *PDH47* (Singha *et al.*, 2017). Initial molecular, biochemical and physiological screening of these transgenic lines under drought stress showed some encouraging results. However, to understand the underlying mechanism of drought stress tolerance/resistance of these transgenic rice lines, a detailed physiological and biochemical analyses are needed. Therefore, keeping these points in mind, the present investigation is envisaged with the following objectives:

1. Genetic transformation of North Eastern (NE) rice cultivars with *PDH47* gene mediated through *Agrobacterium*.
2. Molecular analysis of transgenic events for presence and expression of transgenes in primary transgenics and their progeny.
3. Physiological and biochemical analysis of transgenic rice under drought stress.

Review of Literature…

CHAPTER II

REVIEW OF LITERATURE

Rice is one of the important staple crops for more than 3 billion people worldwide from the point of nutritional security. In terms of cultivation rice stands next to wheat; but rice is the most widely cultivated crop in irrigated areas throughout the world because it requires ample amount of water in different growth stages (Roel *et al.,* 1999). Rice productivity is severely affected by various biotic and abiotic/ environmental stresses. Environmental stresses/abiotic stresses such as drought, heat, salinity and nutrient scarcity can cause average yield loss of more than 50% in most of the cultivated crop species (Wang *et al.,* 2003). The adverse affects of abiotic stresses can be seen at the both cellular and molecular levels because of which the normal functioning of the plant such as growth, development and yield are compromised. Changes in molecular level results in differential expression (up- and down-regulation) of variety of genes; thereby enhances the plant adaptability to such unfavourable conditions. The genes which are differentially expressed in response to abiotic/biotic stresses are known as stress responsive genes. Therefore, a clear cut understanding of such molecular changes in response to abiotic stresses will tremendously help the agricultural scientists/plant breeders to develop abiotic stress tolerant/resistant crop plants. Moreover, food security has become one of the major issues, in the current scenario for the ever growing world population. Besides that, the extreme weather conditions, reduced availability agricultural lands, increased incidence of abiotic stresses are the limiting factors of crop production. Therefore, it has became imperative to develop sustainable abiotic stress tolerant/resistant crop species to mitigate the global food demand (Takeda and Matsuoka, 2008; Newton *et al.,* 2011).

2.1. Drought stress

In case of agricultural science, stress is an altered physiological condition caused either by biotic/abiotic factors which disrupts the normal functioning of the plants. Strain is any physical and chemical change incurred due to stress (Gaspar *et al.,* 2002). Plants which are growing in nature are frequently exposed to a variety of stresses such as drought, low temperature, salt, flood, heat, oxidative stress and heavy metal

toxicity. Among these abiotic stresses, drought stress is one of the most prevalent stresses that occur in regions of scanty rainfall. In agriculture, drought is defined as a period of sufficiently prolonged dry spell in an area under question and results in the lack of crop growth and production. India accounts for the largest share (59%) of the total drought-prone rice cultivating area in Asia and most of these areas are rain fed (Ravikumar *et al.,* 2014). Generally, the rainfall distribution pattern in Western and North-Eastern (NE) India during the southwest (SW) monsoon is totally different. Drought prevalence in India varies from region to region; NE region (especially Assam and Meghalaya) encounter drought once in 15 years as compared to the Western region (especially Rajasthan and Gujarat) where it is more frequent and occurs once in every 2-3 years (Parida & Oinam, 2015). Unfortunately, many districts of Assam (including Bongaigaon, Cachar, Dhubri, Goalpara, Golaghat, Hailakandi, Jorhat, Kampur, Karbi-Anglong, Kokrajhar, Lakhimpur, Morigaon, Nagoan, Nalbari, Sonitpur) and Meghalaya had experienced drought consecutively during 2005 and 2006. Subsequently in 2009, all the seven states of the NE region encountered severe drought situation.

2.2. Impacts of drought on crop plants

Drought has a significant impact on the growth, development and production of almost all the crop species. Severe desiccation and transpiration from the leaf surface is a common phenomenon during drought stress, as a result of which the plant metabolisms, cell structure and eventual cessation of various enzyme catalyzed reactions are grossly disrupted (Smimoff, 1993; Jaleel *et al.,* 2007d). Drought stress is mostly characterized by diminished leaf water potential and turgor loss, closure of stomata and decrease in cell enlargement and growth. Drought stress inhibits cell enlargement more than cell division. It reduces plant growth by affecting various physiological and biochemical processes, such as photosynthesis, respiration, translocation, ion uptake, carbohydrates, nutrient metabolism and growth promoters (Jaleel *et al.,* 2008a-e; Farooq *et al.,* 2008). Drought stress also reduces the photoassimilation/partitioning of metabolites required for cell division resulting in impaired mitosis, cell elongation and expansion that ultimately result in reduced growth. The major impacts of drought stress on crop plants is discussed below-

2.2.1. Reduction in crop growth and development

The first and foremost effect of drought is the reduction in rate of seed germination and poor stand establishment (Harris *et al.,* 2002). It has also been reported

that drought stress severely reduce germination and seedling stand (Kaya *et al.,* 2006). In rice, occurrence of drought stress during vegetative stage can cause reduced plant growth and development (Tripathy *et al.,* 2000; Manikavelu *et al.,* 2006).

Unavailability of water effects the crop growth not only by restricting the cell division, cell enlargement and differentiation but also by abnormal genetic, physiological, ecological, morphological and complex interaction of all these processes (Farooq *et al.,* 2008). Prevalence of drought during pre-anthesis periods significantly reduces time to anthesis; whereas its occurrence during post anthesis periods results in shortening of grain-filling period leading to triticale genotypes (Estrada-Campuzano *et al.,* 2008). Drought stress during flowering stage leads to barrenness of the grains due to insufficient photoassimilate translocation. Besides that it also prevents peduncle elongation, obstructing exertion of spikelet and thereby causing sterility during the emergence of panicle. The negative impacts of drought in morphological parameters of crop plants is discussed below-

2.3. Morphological parameters

2.3.1. Root Architecture System

The change in root architectural system during drought stress is a common adaptive mechanism shown by almost all the crop plants. The understanding of root architectural system and mechanisms of its development will tremendously help the plant breeders/agricultural scientists to exploit and manipulate different root traits to improve plants adaptation to drought stress and increase yields for the growing global human population (Smith and De Smet, 2012). The root architectural system is influenced by various factors such as soil moisture, temperature, nutrient availability and soil pH (Bao *et al.,* 2014; Robbins *et al.,* 2015) which greatly affects crop growth and yield (Manavalan *et al.,* 2010; Prince *et al.,* 2013). Many of the root characters such as root tip diameter (Haling *et al.,* 2013), morphological plasticity (Lynch, 2007; Forde, 2009), gravitropism (Uga *et al.,* 2013) and rhizosheaths (Delhaize *et al.,* 2012) allow the plants to adapt and respond to various environmental factors and they might be quite useful for improving water use efficiency in different crop species (Fenta *et al.,* 2014). Therefore, it is very important to understand the root architectural system regulating mechanisms for crop improvement (Den Herder *et al.,* 2010; Lynch, 2007). The study of RSA is important for agricultural productivity because most soils have uneven distribution of resources and/or localized depletions that make spatial distribution of the

root system an important determinant of a plant's ability to exploit these resources (Lynch, 1995).

2.3.2. Alteration in root to shoot (R/S) ratio

Rice (*Oryza sativa* L.) plants often alter morpho-anatomical traits, physiological and biochemical processes, gene expression and metabolic regulation networks in response to water-stressed condition (Tripathy *et al.,* 2000; Guo *et al.,* 2006; Kathiresan *et al.,* 2006; Zhou *et al.,* 2007; Ji *et al.,* 2012). Since plants acquire water through roots, any plasticity in root architecture enables plants to respond to a changing environment. For example, root often grows thicker, deeper and larger in response to drought in rice genotypes (Azhiri-Sigari *et al.,* 2000; Asch *et al.,* 2005; Yue *et al.,* 2006; Ji *et al.,* 2012; Lemoine *et al.,* 2013). Plants often re-allocate assimilates from shoot to root under drought stress condition, thereby increasing root extension into deeper soil layers (Rich and Watt, 2013). Previous studies have demonstrated that to some extent, drought stress often induces root growth and root architecture is subsequently modified to enhance water uptake in these plants (Asch *et al.,* 2005; Lemoine *et al.,* 2013). On the other hand, several studies have documented that roots growth decreases under drought stress in rice (Azhiri-Sigari *et al.,* 2000; Cui *et al.,* 2008). Such observations suggest that root growth in response to drought stress depends on genotype, degree plus duration of drought stress and rate of stress development. The root to shoot ratio (R/S) and shoot to root ratio are often used to estimate relative biomass allocation between roots and shoots (Wilson 1988; Gowda *et al.*, 2011; Poorter *et al.,* 2012). As reviewed by Wilson (1988), several factors influence biomass allocation, such as deficits of water and major inorganic nutrients, light and carbon dioxide, defoliation and root pruning etc. Distribution of carbohydrates between shoots and roots may be associated with the change in R/S. Farrar (1996) proposed that among all the carbohydrate, sucrose might play a key role in biomass partitioning between shoots and roots. Therefore, source-to-sink transport of sucrose is one of the major determinants of plant growth and relies on the controlled distribution of sucrose (Lemoine *et al.,* 2013). Sucrose and its metabolism are inextricably linked to cellular biosynthesis, signal transduction, and the biosynthesis of osmo-protective substances in plants (Geigenberger *et al.,* 1997; Chiou and Bush 1998; Vargas *et al.*, 2007; Wind *et al.,* 2010; Hammond and White 2011).

2.4. Effect of drought on physiological characteristics of cultivated rice plants

Drought stress significantly affects various physiological processes and induces several physiological responses in plants, which enable them to cope up with such unfavourable environmental conditions. Optimization/standardization of these physiological processes is prerequisite for increased water productivity under water stress (Serraj *et al.,* 2009). The proper understanding of these physiological responses of rice under drought conditions may contribute to ongoing studies on generation of drought resistant/tolerant rice cultivars. An important physiological response of plants to drought is maintaining the turgor pressure by reducing osmotic potential as a tolerant mechanism (Maisura *et al.,* 2014). Water scarcity affects rice physiology in numerous ways like it affects plant net photosynthesis (Centritto *et al.,* 2009; Yang *et al.,* 2014), transpiration rate (Cabuslay *et al.,* 2002), stomatal conductance (Ji *et al.,* 2012; Singh *et al.,* 2013), water use efficiency (Cha-um *et al.,* 2010), intercellular CO_2, photosystem II (PSII) activity (Pieters and Souki, 2005), relative water content (Biswas and Choudhuri, 1984; Pirdashti *et al.,* 2009; Cha-um *et al.,* 2010) and membrane stability index (Kumar *et al.,* 2014). All these parameters reduce under water stress in rice (Farooq *et al.,* 2010; Akram *et al.,* 2013; Ding *et al.,* 2014).

2.4.1. Effect of drought on water relation

A key determinant of plant productivity under drought conditions is water use efficiency (WUE) and it is mentioned as a strategy to improve crop performance under water limited conditions (Araus *et al.,* 2002). Agronomic parameters like photosynthetic rate, relative water content (RWC) and stomatal conductance show strong positive correlations with WUE, whereas transpiration rate expresses negative correlation with WUE under drought in basmati rice varieties (Akram *et al.,* 2013).

When the rice plants are exposed to drought stress the leaf water potential, relative water content and transpiration rate decreases to a significant level (Siddique *et al.,* 2001). Decline of relative water content as a response of stress were reported by several investigators under different stress conditions (Ramanjulu and Sudhakar, 1997; Madhusudan *et al.,* 2002; Turkan *et al.,* 2005; Farooq and Azam, 2006). Hence, higher the leaf temperature results in decrease of stomatal conductance thereby lowering the transpiration rate (Farooq *et al.,* 2008). Abbate *et al.,* (2004) reported that under drought stress condition, the WUE of wheat was greater than non-stressed plants. The higher WUE was due to the closure of stomata which reduces the

rate of transpiration. Thus, as a whole, drought stress disturbs the water relations in plant and the opening and closing of stomata thereby reducing the growth of plant.

2.4.2. Chlorophyll content

Chlorophyll is one of the major chloroplast components for photosynthesis and relative chlorophyll content has a strong relationship with photosynthetic rate. The reduction in leaf chlorophyll content under drought stress has been considered a typical symptom of oxidative stress which can result in pigment photo-oxidation and chlorophyll degradation. Photosynthetic pigments are essential to plants not only for harvesting light but also for producing/generation of reducing powers. Both the chlorophyll a and b are significantly hampered by soil dehydration (Farooq *et al.,* 2009). Drought stress is known to alter the ratio of chlorophyll 'a' and 'b' and carotenoids (Anjum *et al.,* 2003). Low concentrations of photosynthetic pigments can directly limit photosynthetic potential and thus can reduce primary production. From a physiological perspective, leaf chlorophyll content is a parameter of significant interest in its own right. Studies by majority of chlorophyll loss in plants in response to water deficit occurs in the mesophyll cells with a lesser amount being lost from the bundle sheath cells.

2.4.3. Cell membrane stability

The very first target of many abiotic stresses is the biological membranes. It is generally accepted that the maintenance of integrity and stability of membranes under water stress is a major component of drought tolerance in plants (Bajji *et al.,* 2002). Cell membrane stability, reciprocal to cell membrane injury, is a physiological index widely used for the evaluation of drought tolerance (Premachandra *et al.,* 1991). Moreover, it is a genetically related phenomenon since quantitative trait loci for this have been mapped in drought-stressed rice at different growth stages (Tripathy *et al.,* 2000). Dhanda *et al.,* (2004) showed that membrane stability of the leaf segment was the most important trait to screen the germplasm for drought tolerance.

2.4.4. Effect of drought stress on photosynthesis

Environmental stresses have a direct impact on the photosynthetic apparatus, essentially by disrupting all major components of photosynthesis including the thyllakoid electron transport, the carbon reduction cycle and the stomatal control of the CO_2 supply, together with an increased accumulation of carbohydrates, peroxidative destruction of lipids and disturbance of water balance (Allen and Ort, 2001). Negative

effects of high temperature and vapour pressure deficit on leaf photosynthesis and water use efficiency (the ratio of photosynthetic and transpiration rates) can be partly offset by an increased atmospheric CO_2 concentration. This can be accomplished by maintaining the optimal CO_2 concentration in the sub stomatal chamber at a lower level of stomata opening, resulting in lower rates of transpiration saving water (Condon *et al*., 2002). Drought stress severely hampered the gas exchange parameters of crop plants and this could be due to decrease in leaf expansion, impaired photosynthetic machinery, premature leaf senescence, oxidation of chloroplast lipids and changes in structure of pigments and proteins (Menconi *et al*., 1995).

Stomata are the entrance of water loss and CO_2 absorbability and stomatal closure is one of the first responses to drought stress which result in declined rate of photosynthesis. Stomatal closure deprives the leaves of CO_2 and photosynthetic carbon assimilation is decreased in favor of photorespiration. Considering the past literature as well as the current information on drought-induced photosynthetic responses, it is evident that stomata close progressively with increased drought stress. It is well known that leaf water status always interacts with stomatal conductance and a good correlation between leaf water potential and stomatal conductance always exists, even under drought stress. It is now clear that there is a drought-induced root-to-leaf signaling, which is promoted by soil drying through the transpiration stream, resulting in stomatal closure. The "non-stomatal" mechanisms include changes in chlorophyll synthesis, functional and structural changes in chloroplasts, and disturbances in processes of accumulation, transport, and distribution of assimilates.

2.4.5. Effect of drought stress on stomatal conductance

The capability of plants to survive under stressful conditions is often referred to as stress tolerance and therefore, it represents an important trait in agriculture. For most of the plants, stress tolerance is a ‘developmental or physiological programme’ that requires activation or induction by the stress signals (Luan, 2002).

Stomata serve as both exit and entry points for water vapour and CO_2 respectively. Hence, the rate of transpiration and net photosynthesis depend on controlled stomatal opening and closing which is the first response in plants against water deficit conditions (Cornic and Massacci, 1996; Reddy and Vanaja, 2006). Therefore, it is the major way of regulating water relations and carbon assimilation in plants (Hetherington and Woodward, 2003). It is well known that plants under water

stress conditions shows decreased level of stomatal conductance (Gangadhar Rao and Hebbar, 1996; Cornic, 2000).

Lu *et al.,* (1998) found that the stomatal conductance has a positive relationship with yield and can be used to develop high yielding genotypes. The lower stomatal conductance inhibits water losses, thereby helps the plants to withstand water scarce condition. The term 'water saviours' has been given to these type of plant.

2.4.6. Effect of drought stress on transpiration rate

A plant with a high transpiration rate is known to maintain an efficient energetic flow from the chemical energy production site of PSII to CO_2 assimilation site. The transpiration efficiency is an important indication of water yield variation under drought stress in many crops (Ratnakumar *et al.,* 2009).

An increased transpiration rate was observed in tolerant varieties of blackgram coupled with increased photosynthetic rate, stomatal conductance and per plant yield (Babu *et al.,* 2009).

2.4.7. Water use efficiency (WUE)

WUE is one of the key physiological traits determining plant productivity under limited water supply (Sudhakar *et al.,* 2006) and it is mentioned as a strategy to improve the performance of crop under drought stress situation (Araus *et al.,* 2002).

In agronomic terms, it is defined as the ratio between total dry matter produced (or yield harvested) and water used (Jones, 1993). In physiological terms, however, WUE is defined as the ratio between the rate of carbon fixed and the rate of water transpired. Agronomic parameters such as photosynthetic rate, relative water content and stomatal conductance show a positive strong correlation with WUE, whereas the rate of transpiration expresses negative correlation with WUE under drought stress in Basmati rice varieties (Akram *et al.,* 2013).

2.5. Biochemical responses of cultivated rice plants under drought stress

Reduced osmotic potential in the cytosol is as a result of the accumulation of inorganic and organic solutes, which leads to the maintenance of turgor pressure under drought stress (Rhodes and Samaras, 1994). This biochemical procedure is a type of osmotic adaptation that strongly depends on the water stress level. Osmotic adaptation occurs *via* the accumulation of glycine betaine, sucrose, proline, and other

solutes in the cytoplasm, promoting water uptake by drying the soil. Proline, an amino acid, is the most widely investigated due to its considerable stress-reducing or function under adverse conditions. Water deficit also induces soluble sugar accumulation (Usman *et al.,* 2013; Maisura *et al.,* 2014; Shehab *et al.,* 2010).

2.5.1. Role of Proline under drought stress

Proline being an osmolyte plays a very crucial role in plants encountering various stress conditions (Verbruggen and Hermans, 2008). The free proline accumulation in plants due to water scarcity was reported for the very first time by Kemble and Mac-Pherson in the year 1954 in rye grasses. Changes in the concentration of proline have been observed in rice exposed to drought stress (Sheela and Alexallder, 1995; Mostajeran and Rahimi-Eichi, 2009; Bunnag and Pongthai, 2013; Kumar *et al.,* 2014; Lum *et al.,* 2014; Maisura *et al.,* 2014). Besides acting as a potent osmolyte, proline plays other important role during drought stress like it as a metal chelator, an antioxidative defence molecule and a signalling molecule (Hayat *et al.,* 2012). Proline accumulation might promote plant damage repair ability by increasing antioxidant activity during drought stress. The proline content in plants encountering drought stress increases significantly compared to the other amino acids because of which it acts as a biochemical marker to select varieties aiming to resist to such conditions (Fahramand *et al.,* 2014).

2.5.2. Role of Glycine Betaine under drought stress

Glycine betaine (N, N, N-trimethyl glycine) is one of the most extensively studied quaternary ammonium compounds and compatible solutes in plants, animals and bacteria (Wahid *et al.,* 2007). Many studies demonstrate that glycine betaine plays an important role in enhancing plant tolerance under a range of abiotic stresses including drought (Quan *et al.,* 2004). The introduction of genes synthesizing glycine betaine into non-accumulators of glycinebetaine proved to be effective in increasing tolerance to various abiotic stresses (Sakamoto and Murata, 2002). In addition to direct protective roles of glycine betaine either through positive effects on enzyme and membrane integrity or as an osmoprotectant, glycine betaine may also protect cells from environmental stresses indirectly by participating in signal transduction pathways (Subbarao *et al.,* 2000).

2.5.3. Reactive oxygen species generation and its impact

The generation of reactive oxygen species (ROS) is one of the earliest biochemical responses of plants to biotic and abiotic stresses. The production of ROS in plants, known as the oxidative collapse, is an early event of plant defense response to under drought stress and acts as a secondary messenger to trigger subsequent defense reaction in plants. Reactive oxygen species (ROS), which include oxygen ions, free radicals and peroxides, form as a natural by-product of the normal metabolism of oxygen and have important function in cell signalling. However, during environmental stress such as drought, ROS levels increase dramatically resulting in oxidative damage to proteins, DNA and lipids (Apel and Hirt, 2004). Being extremely reactive, ROS can severely damage plants by increasing lipid peroxidation, protein degradation, DNA fragmentation and finally cell death. Drought stress induces oxidative stress in plants by generation of reactive oxygen species (ROS) (Farooq *et al*., 2009). Drought-induced high production of ROS increases the content of malondialdehyde (MDA). The content of malondialdehyde (MDA) has been counted an indicator of oxidative damage (Moller *et al*., 2007). MDA is considered as a suitable marker for membrane lipid peroxidation. A decrease in membrane stability reflects the level of lipid peroxidation caused by ROS. Moreover, lipid peroxidation is an indicator of the prevalence of free radical reaction in tissues. Oxygen uptake loading on the tissues as both processes generate reactive oxygen species, particularly H_2O_2 that produced at very high rates by the glycollate oxidase reaction in the peroxisomes in photorespiration. The ROS such as O^{2-}, H_2O_2 and $^{\bullet}OH$ radicals, can strongly attack membrane lipids and increase lipid peroxidation (Mittler, 2002).

2.5.4. Role of antioxidative enzymes under drought stress

An imbalance between the quenching and generation of ROS is the most common phenomenon under drought stress (Faize *et al.*, 2011). The ROS include hydroxyl free radicals, singlet oxygen, hydrogen peroxide, and the superoxide radical, and they denature proteins, cause lipid peroxidation, mutate DNA, disrupt cellular homeostasis, and cause cellular oxidative damage. A complex antioxidant system containing enzymatic antioxidants and nonenzymatic molecules protects plants against the adverse effect of ROS. Glutathione (GSH) and ascorbate (AsA) serve as nonenzymatic antioxidants within the cell. Enzymatic antioxidants include catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX), superoxide dismutase

(SOD), guaiacol peroxidase (GPX), ascorbate-glutathione cycle enzyme, monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) (Noctor and Foyer, 1998). These antioxidants are vital ROS-scavenging components in crops and their expression increases drought tolerance in rice (Wang *et al.,* 2005). Increasing levels of drought stress in rice lead to increases in the activity of APX, GSH, AsA (Selote and Khanna-Chopra, 2004), GR, MDHAR, SOD, DHAR (Sharma and Dubey, 2005), CAT and phenylalanine ammonia lyase (Shehab *et al.,* 2010). The trend for these antioxidant defence enzymes to increase their activity demonstrates their protective activity to counteract the oxidative injury induced by drought stress in rice. The activities of CAT, POD, and SOD can effectively decrease ROS, which ultimately diminishes the negative impact of drought on rice (Yang *et al.,* 2014; Lum *et al.,* 2014).

2.6. Molecular mechanism of plant responses during drought stress

As a response to stress caused by diverse environmental factors the higher plants have evolved adaptive mechanisms at the physiological, cellular and molecular levels (Marco *et al.,* 2015). The response of a plant to abiotic stress, first involves the perception of the extracellular stress signal by receptors of the cell, followed by many stress regulatory networks, including signal transduction and transcriptional regulation of stress-responsive gene expression that result in physiological response of tolerance or resistance of the plant to stress (Huang *et al.,* 2012). At the molecular level the response of the plant to abiotic stress, such as drought, comprises the participation of signalling molecules such as hormones, transcription factors and stress-responsive genes coding for proteins with protective roles against stress, including LEA proteins and peroxidases. Therefore, the elucidation of a molecular pathway for plant response to stress is essential to understanding how plants respond and adapt themselves to diverse abiotic stress.

2.7. Approaches for drought stress tolerance

In order to attain the global food security and sustainable crop production to mitigate the demands of the ever growing population crop improvement remains only the option. As such, it can be achieved by three different approaches such as conventional breeding, Marker Assisted Selection and Generic engineering/ transgenic approach.

2.7.1. Classical breeding approach for drought stress

Conventional selection and breeding approaches are the oldest techniques of crop improvement against various abiotic stresses. However, the rate of progress in improving stress tolerance in crops through conventional breeding program is limited because abiotic stress tolerance is a highly complex quantitative trait which is controlled by the expression of several minor genes. Apart from that, the existence of genetic variation at the inter, intra-specific levels and intra-varietal levels makes the selection and breeding processes more difficult for enhanced resistance to any stress (Blum, 1985; Ashraf and Sharif, 1998; Serraj *et al.,* 2005). Moreover, the techniques which are employed for selecting such genetically variant stress tolerant plants are mostly time consuming highly expensive. There is an alternative systematic way to improve the drought stress tolerance by pyramiding the various adaptive traits in one plant genotype. Those adaptive traits may be rapid establishment, phenology, early vigour, root depths and root density, low and high temperature tolerance, root conductance, osmoregulation, sugar accumulation in stems to support the later growth of yield components (Parry *et al.,* 2005). On the other hand, use of marker assisted selection seems to be a more appropriate approach because it enable to dissect quantitative traits into their single genetic components, which makes the selection of drought stress tolerant/resistant plants more easier (Chinnusamy *et al.,* 2005; Hussain, 2006). Lanceras *et al.,* (2004) found that favourable alleles for yield components were located in the region of rice chromosome 1 where QTLs for many drought related traits (root dry weight, relative water content, leaf rolling and leaf drying) were previously identified (Zhang *et al.,* 2001). Despite of theoretical advantages of utilizing MAS to improve quantitative traits during the past decade, the overall impact of MAS on the direct release of drought tolerant/resistant cultivars remains non-significant (Reynolds and Tuberosa, 2008). By summarizing all the reports mentioned earlier, it can be easily perceived that molecular breeding work has not been extended beyond the detection of a given trait under water stress conditions. However, whether QTL identified in a given mapping population will improve the drought tolerance in other high yielding elite genotypes upon introduction is still a great challenge for researchers.

2.7.2. Genetic Engineering approaches for drought stress tolerance

A very few crop varieties have been generated with improved stress tolerance through traditional breeding strategies (Flowers, 2004). Besides that, the

progress made through conventional breeding approaches in developing drought stress tolerant/resistant cultivars is very slow and steady. To overcome such problems, biotechnological tools such as genetic engineering would be an appropriate strategy to target/manipulate specific pathways associated with drought tolerance. Till date the function of about 60,000 rice genes have been uncovered due to the advancement made in whole genome sequencing project of rice through functional genomics approach. Therefore, genetic engineering approaches became the method of choice for improving abiotic stress tolerance/resistance in crop plants through introgression of novel candidate genes that confer the stress tolerance mechanisms. The genes which can confer drought tolerance/resistance in crop plants either involved in signalling and regulatory pathways or encode enzymes that leads to synthesis of osmolytes and osmoprotectants, antioxidants or that encode transcription factors and stress tolerance protein (Wang *et al.,* 2003; Vinocur and Altman, 2005). Transgenic plants have been developed initially with these genes using model plants like *Arabidopsis* and tobacco. However, relatively little work has been done on crop plants. Most successful examples of development of transgenic crops for drought tolerance are due to incorporation of the various transcription factors in different crop species. Over-expression of *DREB1A* cDNA under the control of CaMV35S constitutive promoter in transgenic *Arabidopsis* plants activated the expression of many stress tolerance genes which resulted in increased tolerance to drought, salt loading and freezing (Gilmour *et al.,* 2000; Liu *et al.,* 1998). Stress-inducible expression of *AtDREB1A* transcription factors from *Arabidopsis* greatly improves drought stress tolerance in transgenic *indica* rice (Ravikumar *et al.,* 2014). Similarly, overexpression of *ZFP252*, a TFIIIA-type zinc finger protein gene in rice increased the amount of free proline and soluble sugars elevated the expression of stress responsive genes and enhanced rice tolerance to salt and drought stresses (Xu *et al.,* 2008). Transgenic rice plants overexpressing *DREB1A* transcription factor gene from both *Arabidopsis* and rice and *DREB1B* stress gene from rice under the control of the stress inducible rd29 promoter confer both drought stress and salt stress tolerance (Datta *et al.,* 2012). Similarly, Wang *et al.,* (2016) reported that the *IbZFP1* gene of sweet potato, which encodes a novel Cys2/His2 zinc finger protein which is responsible for regulation of ABA signalling, proline biosynthesis and ROS scavenging and thereby, improves salt and drought tolerance in transgenic *Arabidopsis* plant. Singh *et al.,* (2016) reported that introduction of SbSDR1, a novel transcription factor gene acts as a molecular switch and confers salinity and osmotic stress tolerance

in transgenic tobacco plants under stress conditions. Transgenic rice plants over expressing *TaSTRG* gene from (*Triticum aestivum),* a salt tolerance-related gene showed higher salt and drought tolerance than the control (Zhou *et al.,* 2009). Likewise, Pea DNA helicase 45 promotes salinity stress tolerance in IR64 rice with improved yield (Sahoo *et al.,* 2012). First transgenic plant for drought stress tolerance by overproducing gene encoding proline was reported in tobacco (Kavi-Kishore *et al.,* 1995) and rice (Zhu *et al.,* 1998).

2.8. *PDH47* helicase gene in improving tolerance to abiotic stress in plants

In crop plants encountering drought stress condition, the stress signal is first perceived by membrane bound receptors and activates the downstream signal cascade reaction that leads to expression of various stress responsive genes to provide stress tolerance. Genes from the nucleic acid pathways (mainly helicases) are known to be upregulated in response to various abiotic stresses such as drought, cold, salinity etc. In *Pisum sativum*, a large and complex signalling cascade is activated while encountering drought stress situation which results in the expression of stress-responsive DNA helicase 47 (*PDH47*). The *PDH47* gene belongs to the DEAD-box protein family which is composed of nine conserved helicase domains (Linder *et al.,* 1989; Cordin *et al.,* 2004; Tuteja and Tuteja, 2004a, b). The structural studies of the purified *PDH47* recombinant protein (47 kDa) revealed that it has ATP/Mg^{2+}-dependent ATPase unwinding activities. *PDH47* gene localized mostly in the cytosol and unwinds DNA in the 3'-5' direction using ATP that has multiple functions like efficient translation under stress condition or regulating the DNA/RNA metabolism (Gong *et al.,* 2002; 2005; Vashisht and Tuteja, 2005). The *PDH47* protein also showed up-regulation in the rate of synthesis of some other proteins during the abiotic stress condition in plants. Many studies revealed that *PDH47* gene may act at the translational level to enhance or stabilize protein synthesis during abiotic stress conditions in plants. The enhanced expression of DNA helicases during stress condition might be one of the important survival mechanisms adopted by the crop plants (Vashisht and Tuteja, 2005; Vashisht *et al.,* 2005). It has been reported that the heterologous expression of *PDH47* transcript regulated several endogenous stress-responsive genes in transgenic rice during drought stress. Singha *et al.,* (2017) showed that the over-expression of *PDH47* in the transgenic lines was associated with higher accumulation of osmolytes like proline and retained higher relative water content and low accumulation of H_2O_2 under drought stress.

2.9. Rice transformation

Several genetic transformation methods of rice have been developed over the years. However, there is a restriction that *indica* variety of rice is recalcitrant to high efficiency transformation and some genotypes that could not be tamed by employing the common protocols. Therefore, *indica* rice variety demands an alternative efficient way with better transformation protocols (Bajaj and Mohanty, 2005).

2.9.1. *Agrobacterium* mediated genetic transformation of rice

Rice transformation using *Agrobacterium tumefaciens* is a method of choice due to stable and low copy number integration of transfer-DNA (T-DNA) into the plant chromosome and transfer of larger DNA segments with defined ends (Komari *et al.,* 1996; Agarwal *et al.,* 2002). *Agrobacterium*-mediated transformation is now the preferred method of gene delivery into rice, as this procedure has several advantages, compared to direct DNA uptake procedures (Tyagi *et al.,* 1999; Taylor and Fauquet, 2002). Transgenic rice lines of elite *indica* rice cultivars (IR64, Pusa Basmati-1 and Karnal Local) were generated through *Agrobacterium*-mediated method with a synthetic *cry1Ac* gene driven by the maize ubiquitin-1 promoter and there was enhanced resistance to yellow stem borer (Khanna and Raina, 2002). *Indica* genotypes of rice transformed with the chitinase cDNA through *Agrobacterium*-mediated gene transfer method using mature seed derived calli as explants showed enhanced sheath blight resistance (Nandakumar *et al.,* 2007).

2.9.2. Immature embryo as explants

The choice of tissue as a starting material is very important for an efficient genetic transformation. The various explants employed for rice transformation are mature (Raineri *et al.,* 1990; Sahoo *et al.,* 2011; Basu *et al.,* 2014; Gui *et al.,* 2016) or immature embryos (Hiei *et al.,* 1994; Aldemita *et al.,* 1996), excised stems, leaves, and roots (Chan *et al.,* 1992), shoot apices, roots and scutella (Hiei *et al.,* 1994; Fook *et al.,* 2015). Both the mature and immature explants can be employed as explants for the rice transformation. Mature embryos of japonica rice cv. were considered for the induction of tumorigenic callus tissues with a confirmed status of transformation (Raineri *et al.,* 1990). Excised stems, leaves, and roots can also be used as starting material for rice transformation. Chan *et al.,* (1993) successfully transferred and expressed *uidA* (GUS) driven by α-amylase promoter in a *japonica* rice cv. Tainung 62 employing *Agrobacterium*-mediated transformation system with immature embryos.

2.9.3. Efficient *Agrobacterium* strains

A number of *Agrobacterium* strains have been used for genetic transformation rice which includes LBA4404, EHA101, EHA105, AGL1 etc.Four *indica* rice cultivars were successfully transformed using EHA101 strain harbouring the binary vector pCAMBIA1301 (Lin and Zhang, 2005). Similarly, Rashid *et al.,* (1996) transformed three indica rice cultivars using *Agrobacterium* strain EHA101 harbouring the standard binary vector pIG121Hm. Guruprasad *et al.,* (2012) used *Agrobacterium* strains LBA4404 for successful rice transformation. Sahoo *et al.*, (2011) used *Agrobacterium* strains LBA4404 and EHA105 for introduction of glyoxalaseI gene into *indica* rice cultivar (IR64, CSR10, PB1 and Swarna).

2.9.4. Genotype, medium composition and co-cultivation conditions

Manipulation of plant tissues and cells *in vitro* has been employed to increase the T-DNA delivery and thus enable *Agrobacterium* to transform a wide range of recalcitrant plants (Karthikeyan *et al.,* 2011). The factors include the selection of competent genotypes, composition of the media and co-cultivation conditions. Many researchers have tried different culture mediums and conditions for the inoculation and co-cultivation of *Agrobacterium* to optimize the efficiency of *Agrobacterium*-mediated rice transformation (Karthikeyan *et al.,* 2011). Hiei *et al.,* (1994) used a modified N6 medium containing acetosyringone, 2, 4-D and casamino acids for co cultivation of rice calli with *Agrobacterium*. Browning of tissue or necrosis is reported to be reduced by using a solid co-cultivation medium containing reductants such as ascorbic acid or L-cysteine (Enriquez-Obregon *et al.,* 1999; Olhoft *et al.,* 2001). Acidic pH, incubation temperature of 28°C or less (Alt-Moerbe *et al.,* 1988) and high osmotic pressure (Gandhi and Khurana, 1999; Jain, 1997) are the other important factors for co-cultivation and *vir* gene induction. Several media and treatments for tissue culture and high frequency regeneration of transformed tissues of *japonica, javanica* and *indica* rice cultivars have been reported (Jain, 1997). Water stress treatment (use of 1.6% agar instead of 0.8% agar for medium solidification) resulted in higher frequency regeneration from transformed *indica* rice calli in the absence of hormones was reported by Gandhi and Khurana (1999). According to Aldemita and Hodges (1996), inclusion of a selection agent in the regeneration medium has been reported to favour the production of a large numbers of genetically stable transgenic rice plants.

Hiei and Komari (2008) reported comprehensive, highly efficient protocols using *Agrobacterium tumefaciens*-mediated transformation for a wide range of rice genotypes. They used either immature embryos (*japonica* and *indica* rice) or calli (*japonica* cultivars and the *indica* cultivar, Kasalath) as a starting material for inoculation with *Agrobacterium*. Heat-treatment of immature embryos with followed by centrifugation significantly enhances the efficiency of gene transfer through *Agrobacterium* mediated technique. A single immature japonica or Kasalath embryo will produce between 10 and 18 independent transgenic plants; for other non-Kasalath *indica* varieties, the number of transgenic plants expected will be between 5 and 13. For *japonica* and Kasalath, transformants should be obtained from between 50 and 90% of calli. From inoculation with *Agrobacterium* to transplanting to soil will take 55 days for *japonica* and Kasalath, and 74 days for *indica* other than Kasalath using the callus method (Hiei and Komari, 2008). Sahoo *et al.,* (2011) used Murashige and Skoog (MS) media supplemented with maltose, 0.3g/L casein hydrosylate, 0.6g/L L-proline, 3.0mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.25mg/L 6-benzylaminopurine (BAP) for callus induction from mature seeds of four indica rice varieties IR64, CSR10, PB1 and Swarna. Similarly, Priya *et al.,* (2015) used MS medium supplemented with 30g/L maltose, 13.5µM of 2, 4-D and 1.3µM of kinetin for genetic transformation of mature seeds of IR64.

Materials and Methods…

CHAPTER III

MATERIALS AND METHODS

3.1. Chimeric gene construct used

The plasmid *pCAMBIA1301* harbouring *PDH47* gene driven by Cauliflower Mosaic Virus 35S (CaMV35S) used in the current study was obtained from ICGEB, New Delhi. The construct contains hygromycin phosphotransferase (*hpt*II) and *GUS* as a plant selectable marker gene and reporter gene, respectively. The *hpt*II gene was driven by CaMV35S promoter and 35S polyA terminator whereas *GUS* gene was driven by CaMV35S promoter and nos polyA terminator. The diagram of the gene construct used in the present study is shown in Fig. 3.1.

3.2. Bacterial transformation of *p1301-PDH47* vector into *Escherichia coli (E. coli)* DH5∝ strain

3.2.1. Preparation of DH5∝ competent cell

Single colony of DH5∝ strain was inoculated in 5 ml of Luria Bertini (LB) broth and allowed to grow overnight. In the next day, one millilitre of the overnight grown culture was subcultured into 100 ml of LB broth and grown at 37˚C incubator in shaking condition (200 rpm) until OD_{600} reached at 0.4 to 0.5. The cell suspension was then taken out and incubated on ice for 45 minutes. The cells were harvested by centrifugation at 3000 rpm for 15 mins at 4˚C. Then the harvested cells were resuspended in 3-4 ml of 100 mM sterile ice cold $CaCl_2$ and incubated on ice for 1 hours with intermediate swirling at every 15 mins interval. The cells were then again centrifuged at 3000 rpm for 15 mins at 4˚C. The supernatant was discarded and the pellet was resuspended in 2ml of sterile ice cold 100 mM $CaCl_2$ + 15% glycerol and the cells were dispensed in 1.5 ml eppendorf tubes (100 µl/tube aliquot) and stored at -80˚C for further use.

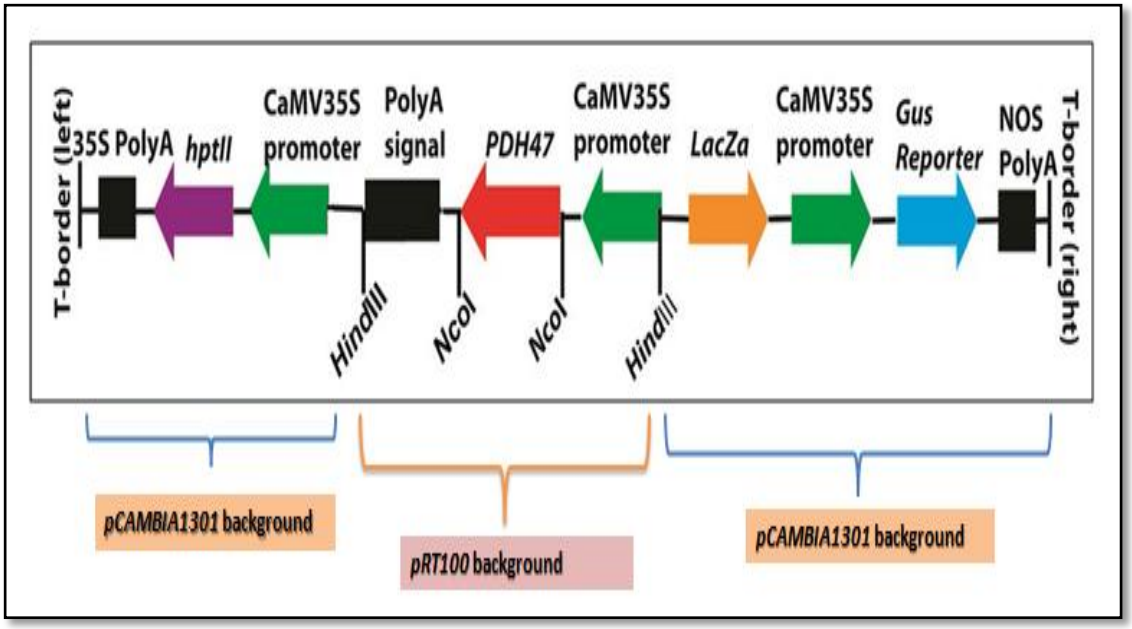


Fig. 3.1: Schematic representation of the gene construct used for rice genetic transformation

3.2.2. *E. coli* transformation

For the bacterial transformation, 1 µl (100ng) of the plasmid DNA was gently mixed with 100 µl of *E. coli* DH5α competent cells and kept on ice for 30 minutes. The ice-cold tubes containing the cells were heat shocked at 42°C for 2 minutes and incubated on ice for 5 minutes. After that 1 ml of LB broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.2) was added to each tubes and incubated at 37°C for 1.5 hour at 180 rpm. Finally, 100 µl of the culture was spread on the LB agar plate containing kanamycin antibiotic (50 mg/L). The plates were incubated overnight at 37°C.

3.2.3. *p1301-PDH47* plasmid isolation from transformed colonies of DH5 ∝ strain

Plasmid DNA was isolated from transformed colonies of *E. coli* DH5α strain using alkaline lysis method as described by Sambrook *et al.,* (1989). Single colony was picked with the help of a sterile tooth pick and grown in 5 ml of LB broth containing 50 mg/l kanamycin at 37°C for overnight. The cells were harvested by centrifuging at 10,000 rpm for 2 mins at 4°C. The supernatant was discarded and the cell pellet was resuspended in 200 µl of alkaline lysis solution I and kept on ice for 5 mins. Then, 400 µl of alkaline lysis solution II was added, mixed well and the tubes were kept on ice for another 10 mins. After lysis, 300µl of alkaline lysis solution III was added and mixed well by gentle inversion and kept on ice for 5 mins. The compositions of solution I, II and III are given in Appendix I. The mixture was centrifuged at 12,000 rpm for 10 mins and supernatant was collected in a fresh 1.5 ml eppendorf tube. Equal volumes of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed gently. The mixture was then centrifuged at 12,000 rpm for 10 mins and the aqueous phase was collected carefully. Equal volumes of chloroform: isoamyl alcohol (24:1) was added to the aqueous phase and centrifuged at 12,000 rpm for 10 mins at 4°C. The aqueous layer was collected and equal volumes of isopropanol was added and kept at -20°C for 1 hr. The plasmid DNA was pelleted down by centrifuging at 15,000 rpm for 15 min at 4°C. The pellet was washed with 70% ethanol and air-dried. The pellet was dissolved in 20 µl of TE buffer (10 mM Tris-Cl and 1 mM Na_2EDTA, pH 8.0) containing 1µl RNAse (10mg/ml). The mixture was incubated at 37°C for 20 mins. Finally the samples were stored at -20°C for further use. The quality and quantity of plasmid DNA was confirmed by resolving in 0.8% agarose gel and documented.

3.2.4. Confirmation of the presence of desired plasmid

For confirmation of the presence and integrity of the desired plasmid *p1301-PDH47* two methods were used.

3.2.4.1. Confirmation with restriction digestion

One µg of the plasmid DNA was digested with *Nco*I (Promega, Fitchburg, Wisconsin, USA) in appropriate buffer and incubated at 37°C for 2 hrs. The reaction set up for restriction digestion is shown in Table 1. The reaction was stopped by incubating the mixture at 65°C water bath for 20 min followed by quenching on ice. The digested product was resolved on 0.8% agarose gel and documented.

3.2.4.2. Confirmation using PCR analysis with gene specific primer

PCR was performed for *PDH47* (gene of interest) as described by Sambrook *et al.,* (1989).

Details of primer sequences and PCR as well as RT-PCR assay conditions were presented in Table 2. The PCR was performed using Eppendroff mini thermal cycler. Amplified PCR product was resolved on 1.0% agarose gel and documented using Gel Doc System (BioRad, USA) and documented.

3.3. Mobilization of *p1301-PDH47* plasmid into *Agrobacterium tumefaciens*

The *p1301-PDH47* plasmid harbouring the *PDH47* gene was mobilized into electro competent *Agrobacterium* strain LBA4404.

3.3.1. Preparation of electro-competent cell of *Agrobacterium* strain LBA4404

For the preparation of the electro-competent cells of LBA4404 strain, a single colony of LBA4404 strain was inoculated into 10 ml of MGL (Appendix II) medium containing kanamycin (50mg/L) and rifampicin (10 mg/L) as a starter culture. The culture was grown at 28°C for overnight. On the next day, 3 ml from the starter culture was subcultured into 100 ml of fresh MGL media containing 50mg/L kanamycin 10 mg/L rifampicin and allowed to grow at 28°C until OD_{600} reached at 0.3-0.4. The cells were incubated on ice for 30 mins. The cells were harvested by centrifugation at 5500 rpm for 10 mins at 4°C. The cells were washed two times with 20 ml of ice cold sterile water followed by two times washing with ice cold 10% glycerol. Finally the pellet was resuspended in 1ml of 15% glycerol by gentle swirling and aliqouted in 1.5 ml eppendorf tubes (200 µl/tube) and stored at -80°C until further use.

Table 1. Restriction digestion of plasmid DNA (pCAMBIA1301-PDH47) with *Nco*I restriction enzyme

Reaction components	***Nco*I (20 U/µl)**	
	Undigested (µl)	**Digested (µl)**
Plasmid DNA (2 µg/µl)	1.0	1.0
10X Buffer	2.0	2.0
Restriction enzyme	-	0.5
Sterile water	17	16.5
Total volume	**20**	**20**

Table 2. Details of the primer sequences and PCR assay conditions

SL. No.	Name of the gene	Forward and Reverse primer sequence	Size of the amplicon	PCR Profile
1.	*PDH47*	PDH47 F(5'CAACAGGCTCAGTCTGGAACGG3') PDH47 R(5'GCTGCACATCAATACCACGAGCC3')	796 bp	94˚C for 4 min:1 cycle 94˚C for 1 min: 30cycle 60˚C for 1 min:30 cycle 72˚C for 1 min:30 cycle 72˚C for 10 min:1 cycle
2.	*HPT*	F(5'GGTCAAGACCAATGCGGAGC3') R(5'GCTGCGCCGATGGTTTCTAC3')	663 bp	94˚C for 4 min:1 cycle 94˚C for 1 min: 30cycle 60˚C for 1 min:30 cycle 72˚C for 1 min:30 cycle 72˚C for 10 min:1 cycle
3.	*qPDH47*	F(5'GAGGGACCAGTGTTCGTGAG3') R(5'ACGACCAGGGGTACCTACAA3')	74 bp	98˚C for 5 min: 1 cycle 98˚C for0.05min:40cycle 60˚C for0.10min:40cycle 72˚C for0.20min:40cycle 65˚C for0.05 min:1 cycle 95˚C for 0.5 min:1 cycle

3.3.2. *Agrobacterium* transformation of *p1301-PDH47* plasmid

The *p1301-PDH47* plasmid was mobilized into *Agrobacterium* strain LBA4404 by electroporation. For electroporation, 1 µl (100 ng/µl) of plasmid DNA was added to the 200 µl of the electro-competent cell and transferred into an electro cuvette. Electroporation was performed in MicroPulser^{TM} (BioRad, USA) as per the manufacturer's instruction. 1 ml of MGL medium was added into the electro cuvette, mixed and transferred to an eppendorf tube and cultured at 28°C for 2 hrs. 100 µl of the culture was spread on MGL agar containing kanamycin (50 mg/L) and rifampicin (10 mg/L) and incubated at 28°C for 36 hrs.

3.3.3. Colony PCR for confirmation of positive *p1301-PDH47* colonies

A bacterial suspension was prepared for colony PCR. A single transformed colony of LBA4404 was picked up with the help of a sterile toothpick and added to 100µl of sterile distilled water. The mixture was heated at 95°C for 5 min followed by centrifugation at 12,000 rpm for 2 mins. The supernatant was collected and 5µl of it was used as the DNA template. PCR was performed for *PDH47* gene using gene specific primers. PCR analysis was carried as mentioned in section 3.2.4.2.

3.4. Genetic transformation of *indica* rice cultivars

Genetic transformation of North Eastern *indica* rice cultivars was performed using *Agrobacterium* mediated genetic transformation method.

3.4.1. *Indica* rice cultivars

North Eastern *indica* rice cultivar Ranjit (obtained from RARS, Titabor, Assam Agricultural University, Jorhat, Assam) and IR64 were used as control in the current study.

3.4.2. *Agrobacterium* mediated genetic transformation

Agrobacterium mediated genetic transformation of two indica rice cultivars was performed in the current investigation. The details of the procedure is discussed below-

3.4.2.1. Explant selection

Immature embryo (12-14 days after pollination) of Ranjit, IR64 was used as explants in the present study.

3.4.2.2. Explant preparation

Immature seeds were collected at 12 to 14 days after pollination (DAP). The glumes were removed manually with the help of forceps and the immature seeds were surface sterilised with 70% ethanol for 2 mins. Then the immature seeds were thoroughly washed with sterile water inside the laminar air flow cabinet followed by surface sterilization with 0.1% mercuric chloride for 2 mins. Immature seeds were again thoroughly washed with sterile distilled water for 4-5 times. Immature embryos were isolated from the seeds with the help of forceps and transferred to 0.8% (wt/volume) agar plate (Appendix III). After isolation, all the immature embryos were taken in a 1.5 ml eppendorf tube and immersed in 1 ml of sterile distilled water.

3.4.2.3. Heat pre-treatment of immature embryos

The tubes containing the immature embryos were incubated in a water bath set at 43°C for 30 mins. The tubes were then cooled on ice for 1 min and centrifuged at 1000 rpm for 10 min at room temperature.

3.4.2.4. Preparation of *A. tumefaciens* inoculum

A. tumefaciens strain LBA4404 harbouring *p1301-PDH47* plasmid was cultured on an AB plate (Appendix IV) containing kanamycin (50 mg/L) and rifampicin (10 mg/L) in dark at 28°C for 3 days. The bacterial cells were harvested and re-suspended to 1 ml of AA-infection medium (Appendix V) at a density of 1×10^9 colony-forming units (CFU) per ml (OD = 1.0 at 660 nm).

3.4.2.5. Inoculation and co-cultivation

After heat pre-treatment, the immature embryos were cultured on NB-As medium (Appendix VI) in such a way that the scutellum of the immature embryos remains in upward position. 2µl of *A. tumefaciens* bacterial suspension culture was poured onto each of the immature embryos and kept for 15 min at room temperature. The embryos were then moved into a new (fresh) position of the same plate and incubated in the dark condition at room temperature for 7 days.

3.4.2.6. Resting and selection of transformed calli

After co-cultivation, the elongated shoots were removed from immature embryos and transferred to the first resting medium CCMC (Appendix VII) with scutellum facing upward and incubated in dark conditions at 28°C for 10 days. Each of the enlarged embryos were then cut into 5-6 pieces using a surgical blade and

transferred to second resting medium CCMC and incubated at 28°C for 10 days. The enlarged embryos in the second resting medium were further made into four smaller pieces and transferred to the first selection medium CCMCH30 (Appendix VIII) and incubated under dark conditions at 28°C for 10 days. The proliferated calli were then transferred to second selection medium CCMCH30 and incubated under dark conditions at 28°C for 7 days. The proliferated hygromycin resistance calli were transferred to pre-regeneration medium NBPRCH30 (Appendix IX) and incubated under continuous illumination at 28°C for 7 days.

3.4.2.7. Regeneration, rooting and hardening

The proliferated hygromycin resistant calli from the pre-regeneration medium were transferred to regeneration medium RNMH30 (Appendix X) and incubated under continuous illumination at 28°C for 14 days. The regenerated shoots were removed from the callus and transferred to half strength MS medium and incubated under continuous illumination at 28°C for 14 days for root induction. Plantlets with well established roots and shoots were transferred to pots containing sterilized soil and grown in green house under controlled environmental conditions. Hoagland's solution was applied at regular interval to provide necessary nutrients for good growth.

3.5. Molecular analysis of putative T_0 transgenic plants

PCR analyses of the putative T_0 transgenic lines were performed using gene specific primers.

3.5.1. Genomic DNA isolation

Genomic DNA from putative transgenic rice lines was isolated following the protocol of Doyle and Doyle (1990). 300-500 mg of leaf tissue was homogenized in liquid nitrogen and mixed in 400 µl of pre-heated (65°C) DNA extraction buffer (0.1 M Tris-Cl, 20 mM Na_2EDTA, 1.4 M NaCl, 20% CTAB, 0.2% β-mercaptoethanol, pH 8.0) in 1.5 ml sterile eppendorf tube and incubated at 65°C for 45 mins in water bath with intermediate tapping. Then tubes were brought to room temperature and centrifuged at 12,000 rpm for 10 mins. Upper phase was collected in a new eppendorf tube and equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added. The contents were gently mixed by inverting the tubes and were centrifuged at 12,000 rpm for 10 mins. Upper aqueous phase was taken in a new eppendorf tube and equal volume of Chloroform: Isoamyl alcohol was added. Contents were again centrifuged at 10,000 rpm

for 5 mins. Upper aqueous phase was transferred to a new eppendorf tube and double volume of chilled ethanol and 1/10th volume of 3 M Sodium acetate (pH 5.2) were added. This mixture was kept at -20ºC for 1 hour to allow complete precipitation of DNA and then centrifuged at 12,000 rpm for 10 mins to pellet down the DNA. The pellet was washed with 70% (v/v) ethanol for 5-10 mins and air-dried. Finally, the DNA pellet was dissolved in 30 µl TE buffer (10 mM Tris-Cl and 1 mM Na_2EDTA, pH 8.0). The quality and quantity of genomic DNA was checked by resolving on 0.8% agarose gel.

3.5.2. PCR confirmation

The genomic DNA thus isolated from putative T_0 transgenic lines were subjected to PCR analyses using gene specific primers to amplify the sequences of *PDH47* (gene of interest), *HptII* (antibiotic selection gene), as described in section 3.2.4.2. The amplified products were resolved on 1.2% agarose gel and documented.

3.6. Gene expression analysis in leaf and root tissues of T_3 homozygous transgenic lines through quantitative real time PCR (qRT-PCR)

Three T_3 homozygous transgenic lines (namely ASD16-46/1, ASD16-66/1 and ASD16-68/1) previously developed in the Department of Agricultural Biotechnology was used in the current study. For qRT-PCR analysis RNA was extracted from the leave samples of T_3 transgenic rice lines following the Trizol® method.The cDNA was synthesized using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Clontech, USA: Catalogue No. RR04A) and real time PCR protocol (Table 3) was followed according to manufacturer's instructions given in SYBR® Premix ExTaq™ (Tli RNase H Plus) (Clontech, USA: Catalogue No. RR820A) as shown in The PCR was carried out in Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). All quantitative real-time PCR experiments were performed twice using two biological replicates and each reaction was run in triplicate using the designed gene specific primers.

3.7. Detail biochemical and physiological analyses of three T_3 homozygous transgenic lines under drought stress

Three T_3 homozygous transgenic lines (previously developed in the department of Agricultural Biotechnology) were subjected to drought stress for 18 days in net house condition. A detail physiological and biochemical analyses were performed using three 75 days old T_3 homozygous transgenic (namely ASD16-46/1, ASD16-66/1

Table 3. PCR conditions for real time PCR

SL. No.	Temperature (°C)	Duration (sec)	Cycles	Functions
1	95	30	1	Initial hold
2	95	5	40	2 Step PCR
3	60	30	40	Dissociation

and ASD16-68/1) lines to evaluate their performance under drought stress condition as compared to their non-transgenic counterparts. All physiological and biochemical parameters such as chlorophyll content, water retention capacity, relative water content, electrolyte leakage, malondialdehyde content, glycine betaine content, proline content, net photosynthetic rate, transpiration rate, stomatal conductance and water use efficiency, estimation of antioxidant enzyme activities such as hydrogen peroxide activity, catalase activity assay and ascorbate peroxidase activities were analyzed. Besides that, root architecture system including root length, root number, root thickness, root biomass, shoot biomass and root to shoot ratio were also analysed during drought stress.

3.7.1. Estimation of chlorophyll content

The chlorophyll content of leaf samples of both transgenic lines and non-transformed rice plants were determined using a destructive method suggested by Arnon (1949) before and after drought stress. Chlorophyll was extracted using 80% acetone and the absorption at 663 nm and 645 nm was measured in a spectrophotometer. By using the absorption coefficient, the amount of chlorophyll was calculated using the formula mentioned below-

$$\text{Chl a} = [12.7(A_{663}) - 2.69\ (A_{645})] \times V/\ (1000 \times W)$$

$$\text{Chl b} = [22.7(A_{645}) - 4.68\ (A_{663})] \times V/\ (1000 \times W)$$

$$\text{Total chlorophyll} = [20.2\ (A_{645}) + 8.62\ (A_{663})] \times V/\ (1000 \times W),$$

Where V= Final volume of 80% acetone extract.

W = Fresh weight in grams of tissue.

The data were analyzed using student t-test and finally data were represented graphically.

3.7.2. Estimation of leaf relative water content (RWC)

The leaf water relations was estimated as relative water content (RWC), water saturation deficit (WSD) and relative saturation deficit (RSD) as described by Turner (1986) and Ashraf *et al.*, (1996). The fully expanded second leaf of the main stem of transgenic lines and non-transformed rice plants before and after drought stress were used for RWC estimation. The leaves were excised from the stem in the morning and fresh weight (FW) was taken immediately. The freshly weighed leaves were kept in

falcon tubes containing 10 ml of distilled water at room temperature for about 12 hours. The leaves were taken out from the tubes by draining out the water from the surface and then weighed to determine the turgid weight (TW). After measuring the TW the leaves were covered with aluminium foil and kept in an oven at 70°C for 48 hours and again the leaf weight was measured to determine the dry weight (DW).

The RWC was calculated using the formula mentioned below-

RWC% = [(FW-DW)/ (TW-DW)] × 100.

The WSD was calculated as follows:

WSD = 100-RWC%

The data were analyzed using student t-test and finally data were represented graphically.

3.7.3. Estimation of water retention capacity (WRC)

The water retention capacity of the leaves of both transgenic lines and non-transformed rice plants were determined following the protocol of Worku (1995) before and after drought stress. The plants (both transgenic and non-transformed) grown in pots were covered with a black plastic sheet during night to prevent water loss due to water evaporation. Simultaneously, one leaf per branch (3 replicates) was detached and weighed immediately. The leaves were kept at room temperature (20-25°C) for free transpiration. The weights of these excised leaves were recorded continuously for eight hours at every one hour interval and once again after 24 hour.

The WRC was calculated as the relative decrease in weight in percentage per hour using the formula-

= (Fresh weight of the excised leaf × 100)/ Fresh weight of the leaf after 8 hour and 24 hour of free transpiration.

The data were analyzed using student t-test and finally data were represented graphically.

3.7.4. Estimation of proline content

Proline content of both transgenic lines and non-transformed rice plants determined before and after drought stress by following the protocol suggested by Bates *et al.*, (1973). 0.5g of leaf samples of both transgenic lines and non-transgenic rice plants were homogenized with 5 ml of 3% aqueous sulfosalicylic acid and the

homogenate was centrifuged at 12,000 rpm for 10 min. 2 ml of supernatant was mixed with 2 ml of glacial acetic acid and 2 ml of acid ninhydrin solution. The resultant mixture was boiled at 90°C for 1 hour in a water-bath and then transferred to ice-bath just for a few second in order to stop the reaction mixture. The colour thus developed was extracted with 4 ml of toluene and finally, the absorption of the chromophore was measured at 520 nm. Proline concentration was calculated by using a calibration curve developed with the proline standard and the proline concentration was expressed in µmoles/g of tissue with the help of the following formula-

µmoles of proline/g of tissue = [(µg proline/ml × ml toluene)/115.5] × 5/ g sample, where 115.5 is the molecular weight of proline.

The data were analyzed using student t-test and finally data were represented graphically.

3.7.5. Estimation of Malondialdehyde Content

Malondialdehyde content of both transgenic lines and non-transformed rice plants was determined according to the protocol of Quan *et al*., (2004) before and after drought stress. Leaves of both transgenic lines and non-transformed plants before and after drought stress were homogenized in 5 ml of 10% trichloroacetic acid (TCA), and centrifuged at 12,000 × *g* for 10 min. Two ml of clear supernatant were added to 4 ml of 0.6% thiobarbituric acid (in 10% TCA) and the reaction mixture was incubated at 100°C in a water bath for 15 min. The reaction was terminated at room temperature, and the reaction mixture was centrifuged at 12,000 × *g* for 10 min, after which the absorbance of the supernatant at 450, 532, and 600 nm was determined with a spectrophotometer, respectively and the final concentration of MDA content was calculated using the following formula-

$$C\ (\mu molL^{-1}) = 6.45(O.D_{532} - O.D_{600}) - 0.56\ (O.D_{450})$$

The data were analyzed using student t-test and finally data were represented graphically.

3.7.6. Estimation of electrolyte leakage (EL)

The percentage of ion leakage was determined for both transgenic lines and non-transformed rice plants following the method of Kaya *et al*., (2003) before and after drought stress. Twenty leaf discs from transgenic and non-transformed rice plants collected before and after drought stress. Then the leaf samples were kept in water bath

at 55°C for 30 mins and the initial electrolyte conductivity (EC_1) was measured by electrical conductivity meter. The same samples were again boiled at 100°C in a boiling water bath for 10 mins and the final electrolyte conductivity (EC_2) was measured again by electrical conductivity meter. The electrolyte leakage was calculated by using the formula given below-

$$\text{Electrolyte leakage \%} = [1- (EC_1 /EC_2)] \times 100$$

The data were analyzed using student t-test and finally data were represented graphically.

3.7.7. Estimation of Glycine Betaine (GB) Content

Quaternary ammonium compound of both transgenic lines and nontransformed rice plants was measured as Glycine Betaine according to the protocol of Grieve and Gattan (1983) before and after drought stress. Fresh leaf samples were collected and weighed followed by drying the tissue in hot air oven at 70°C. The dried leaf samples were grinded to fine powder. The leaf powder (0.5g) was shaken mechanically with 20 ml of distilled water for 24 hour at room temperature. The finely ground leaf samples were then filtered and the filtrates were diluted to 1:1 ratio with 2 N H_2SO_4. Aliquots (0.5 ml) were kept in centrifuge tubes and cooled in ice water for 1 hour. Cold KI-I_2 reagent (0.2 ml) was added and the reactants were gently stirred in a vortex mixture. The tubes were stored at 4°C for 16 hour and then centrifuged at 10,000 rpm for 15 min at 0°C. Finally, the supernatant was carefully aspirated with a fine tipped glass tube. The periodic crystals which obtained, were dissolved in 9 ml of 1, 2-dichloroethane and mixed continuously. After 2 hour of mixing, the absorbance reading was measured at 365 nm using a spectrophotometer. Reference standards of GB (0-10 µg/ml) were prepared in 1N H_2SO_4 and their content was expressed in $\mu g.g^{-1}$ FW.

The data were analyzed using student t-test and finally data were represented graphically.

3.7.8. Histochemical detection of hydrogen peroxide (H_2O_2) by DAB assay

Hydrogen peroxide was detected in the rice flag leaf of both transgenic lines and non-transformed rice plants before and after drought stress. The leaves were stained in 1% 3, 3'-diaminobenzidine (DAB) solution (1mg/ml; pH 3.8) (Mostofa *et al*., 2013). After 24 hours of incubation, the leaves were decolorized by immersing them in the fixative solution (ethanol: acetic acid: glycerol) (3:1:1 v/v/v) and boiled for 3-4

hours to remove the chlorophyll content and finally, detection was done for the deep brown polymerized product (for histochemical detection of H_2O_2). After cooling, the samples were visualized under white light and documented.

3.7.9. Gas Exchange parameters

Gas exchange measurements were performed using the top most leaves of both transgenic lines and nontransformed rice plants before and after drought stress. Net photosynthetic rate, stomatal conductance, transpiration rate and water use efficiency (WUE) were assessed on the intact leaves using the LiCor 6400 infrared gas analayzer system (Lincoln, NE, USA) following the manufacturer's protocol under a light intensity of about 1000 $\mu molm^{-2}s^{-1}$, relative humidity of 70% and 30°C to ensure measurements under stable conditions.

The data were analyzed using student t-test and finally data were represented graphically.

3.8.0. Preparation of extracts for assay of antioxidant enzyme activities

The leaf samples of both transgenic lines and non-transformed rice plants were collected before and after drought stress. The leaves were snap-frozen in the liquid nitrogen and finally kept at -80°C for preparation of the enzymatic extract. Leaves (0.5g) were weighed and finely ground in liquid nitrogen by using chilled mortar and pestle. The frozen powder of the ground leaves were homogenized in 100 mM EDTA, 1% (w/v) PVP, 0.5% (v/v) Triton X-100, 5 mM ascorbate and 1 mM EDTA. The homogenate was centrifuged at 15,000 g for 10 min at 4˚C and the supernatant was collected for measurements of antioxidative enzyme activities.

3.8.0.1. Ascorbate peroxidase assay

Ascorbate peroxidase activity was determined following the method described by Nakano and Asada (1981). The activity of ascorbate peroxidase was obtained by adding 600 µl of enzymatic extract (Section 3.8.0) into a 3 ml of reaction mixture solution containing 50 mM Potassium Phosphate buffer, 0.5 mM ascorbate, 0.1 mM H_2O_2 and 0.1 mM EDTA. The oxidation of ascorbate was monitored at 290 nm in a UV spectrophotometer. The molar extinction coefficient was taken as 2.8 $mM^{-1}cm^{-1}$ and the results were expressed as (units/min/gFW).

The data were analyzed using student t-test and finally data were represented graphically.

3.8.0.2. Catalase assay

Catalase activity was determined following the protocol of Luck (1974). The activity of catalase was obtained by adding 100 µl of enzymatic extract (section 3.8.0) into a 3 ml of reaction mixture solution containing 0.5 ml of 0.2 M Phosphate buffer (pH-7.0) and 0.3 ml of (v/v) H_2O_2 and the final volume was made upto 3 ml by adding distilled water. The reaction was started by adding the enzyme and then, the absorbance readings were taken at 240 nm at 0 min and 3 min on UV-Vis Spectrophotometer. The molar extinction coefficient of H_2O_2 at 240 nm was taken as 6.93×10^{-3} $mM^{-1}cm^{-1}$ and the results were expressed as (units/min/gFW).

The data were analyzed using student t-test and finally data were represented graphically.

3.9.1. Study of root architecture system

Root architecture system of both transgenic lines and non-transformed rice plants before and after drought stress were studied by using both basket and PVC pipes methods (Shashidhar *et al.*, 2012).

3.9.1.1. Root length

The root length of transgenic lines and nontransformed rice plants before and after drought stress were determined. These plants were uprooted by digging near the base of the plants and root length of each plants were measured in centimetre and data were recorded.

3.9.1.2. Total number of roots

Total number of roots per plant of both transgenic lines and nontransformed rice plants before and after drought stress at crown region were counted and recorded.

3.9.1.3. Root thickness

The root thickness of both transgenic lines and nontransformed rice plants before and after drought stress was measured using screw gauge and recorded in' mm'.

3.9.1.4. Root dry weight

The roots of both transgenic lines and nontransformed rice plants before and after drought stress were determined. The roots were removed from the shoots and

dried in a hot air oven at 80°C for 48 hour (Until constant weight was obtained) and weight was recorded in gram (g).

3.9.1.5. Shoot dry weight

The oven-dried shoots of both transgenic lines and nontransformed rice plants before and after drought stress were weighed and recorded in gram (g).

3.9.1.6. Root: Shoot ratio

The root weight of plant was recorded as mentioned in section 3.9.1.4. The shoot weight was recorded separately after drying the shoot portion in hot air oven at 80°C for 48 hour until constant weight was obtained and finally, the root: shoot ratio was calculated.

3.9.2. Extraction of Protein Samples for Western Blotting

The leaf samples from both transgenic lines and nontransformed rice plants before and after drought stress were collected and snap-frozen in liquid nitrogen and stored at -80°C for further use. By using mortar pestle, the leaf protein was isolated in 1 ml of ice cold protein extraction buffer (0.1 M Tris-Cl pH-7.6, 0.5 M NaCl, 1 mM EDTA and 50 μM β-mercaptoethanol which is added freshly) and finally the soluble protein was recovered by centrifugation at 10,000 rpm for 10 mins at 4°C. Aliquots of the protein were taken for SDS-PAGE analysis. The total soluble protein content was also measured following the Braford's protocol.

3.9.2.1. SDS-PAGE Electrophoresis

An equal quantity (30 μg) of protein extracted from the leaves of both transgenic lines and nontransformed rice plants were loaded in 12% polyacrylamide gel and allowed to separate according to their molecular weight for 2-3 hours at 65 volt. Finally proteins were transferred from the gel to the nitrocellulose membrane using 1X SDS transfer buffer (appendix XI) for Western Blotting.

3.9.2.2. Western blot analysis

SDS-PAGE separated proteins were transferred to nitrocellulose membrane (Amersham Protran Premium 0.45μm Nitrocellulose membrane) using mini trans-blot electrophoretic transfer cell apparatus (BIORAD, USA). After resolving the protein on 12% acrylamide gel, the stacking gel was removed and equilibrated in transfer buffer for 10-15 mins followed by washing in sterile water before assembling

the unit. Nitrocellulose membrane was cut to the size of the gel. Whatman filter paper was equilibrated in transfer buffer for 5 mins. After assembling (the membrane, gel between filter paper and pads) electro-blotting was performed at 65V for 2 hours. The membrane was removed from the apparatus and washed with Tris buffer saline tween-20 (TBST) (Appendix XII). Then the membrane was kept in 5% skimmed milk in TBST solution for one hour at room temperature with slow shaking. After washing the membrane three times in TBST for 5 mins it was incubated in TBST solution containing primary antibody (Anti-Rubisco, RbcL, AS03 037, Agrisera antibody) at 1:5000 dilution for 1 hour at room temperature. After three washes with TBST for 5 mins each, the membrane was incubated with secondary antibody (Anti-Rabbit IgG **(**H&L), HRP conjugated (Product code: AS09 602, Agrisera antibody) (1:10,000 dilution) for one hour at room temperature with slow shaking. After washing with TBST solution for five mins in TBST, AEC substrate (Product code: 152224, Merck), was added. The membrane was dried on Whatman paper and dark brown bands documented.

Results and Discussions…✍

CHAPTER IV

RESULTS AND DISCUSSIONS

Plants encounter a wide range of environmental stresses throughout its growing period. To increase their tolerance plants have developed several sophisticated defense mechanisms that involve both physiological adaptation and changes in interactive molecular and cellular level. The responses of plants to different abiotic stresses are multigenic and the molecular mechanisms underlying these are not well understood. As abiotic stress affects the cellular gene-expression machinery, it is likely that the molecules involved in nucleic acid processing including helicases, may be affected. Helicases are the enzymes that catalyze the unwinding of double stranded nucleic acids. They generally function *in vivo* as integral parts of macromolecular machines engaged in the physiological processes of nucleic acid metabolism and rearrangement that accompany the cell cycle and the events of gene expression. These processes include replication, recombination, repair, RNA transcription, rearrangement and splicing. In the present study, an attempt was made to introduce drought responsive *PDH47* gene into two *indica* rice cultivars (Ranjit and IR64) of North Eastern India through *Agrobacterium* mediated genetic transformation.

The prime objective of the proposed study was to develop transgenic NE rice cultivars harbouring *PDH47* gene for drought tolerance and detail physiological and molecular screening of all the transgenic rice plants previously developed in the Department of Agricultural Biotechnology, AAU, Jorhat-13 harbouring the same gene. In addition, the homozygosity statuses of the previously developed transgenic plants were not thoroughly studied. Considering the time consuming process in obtaining biosafety related permission for conducting field trials and directly using the cultivars for crop improvement program, back cross breeding program was not adopted in the present study.

4.1. *Agrobacterium* (LBA4404 strain) transformation using *p1301-PDH47* plasmid

The binary plasmid *p1301-PDH47* gene was mobilized into *Agrobacterium tumefaciens* strain LBA4404 through electroporation and the transformed colonies were observed after 2 days in the MGL agar plate containing 50

mg/L kanamycin and 10 mg/L rifampicin antibiotics. No colonies were observed in the control plate (Plate 1a and 1b). Plasmid DNA was isolated from the transformed colonies of *Agrobacterium* and PCR was performed using gene specific primer which confirmed the presence of 796 bp amplicon of *PDH47* gene (Plate 2). Restriction digestion of p1301-*PDH47* plasmid with *Nco*I (20 U/µl) revealed presence of expected product size of 1.6 kb (Plate 3).

4.2. Genetic transformation of North Eastern *indica* rice cultivars

Genetic transformation of two *indica* rice cultivars (Ranjit and IR64) was performed using immature embryos mediated through *Agrobacterium* following the protocol of Hiei and Komari (2008). Transformation of *indica* rice cultivars is comparatively difficult as compared to that of *japonica* rice cultivars as they are more recalcitrant to tissue culture techniques.

4.2.1. *Agrobacterium* mediated genetic transformation

Agrobacterium mediated genetic transformation is a method of choice because it offers several advantages such as high transformation efficiency, the ability to transfer large pieces of foreign DNA, minimal rearrangement of transferred DNA and insertion of a discrete segment of DNA into the recipient genome at a low copy number (Hiei *et al*., 1994; Kumar *et al*., 2005). There are several reports on successful transformation of *indica* rice cultivars mediated through *Agrobacterium* (Rashid *et al*., 1996; Nayak *et al*., 1997; Khanna and Raina, 1999; Datta *et al*., 2000; Pinto *et al*., 2013; Basu *et al*., 2014). However, the transformation conditions were of minimal standard or were applicable to a few *indica* rice varieties. Lin and Zhang (2005) and Hiei and Komari (2008) have developed efficient *Agrobacterium* mediated transformation protocols for elite *indica* rice cultivars like IR8, IR54, IR64 etc. Generally, both mature and immature embryos were used as explants in both *indica* and *japonica* rice cultivars for *Agrobacterium* mediated genetic transformation (Chan *et al*., 1992; Khanna and Raina, 1999; Hossain *et al*., 2009; Sahoo *et al*., 2011). Most of the available reports on *Agrobacterium* mediated genetic transformation of rice using different genes indicated that immature embryos showed better transformation efficiency as compared to the mature seeds in case of *indica* rice cultivars (Hiei and Komari,2008). In the present study, genetic transformation of NE *indica* rice cultivar Ranjit (obtained from RARS, Titabor, Assam Agricultural University, Jorhat-13, Assam) and IR64 (as control) was performed through *Agrobacterium* mediated procedure harbouring the binary vector

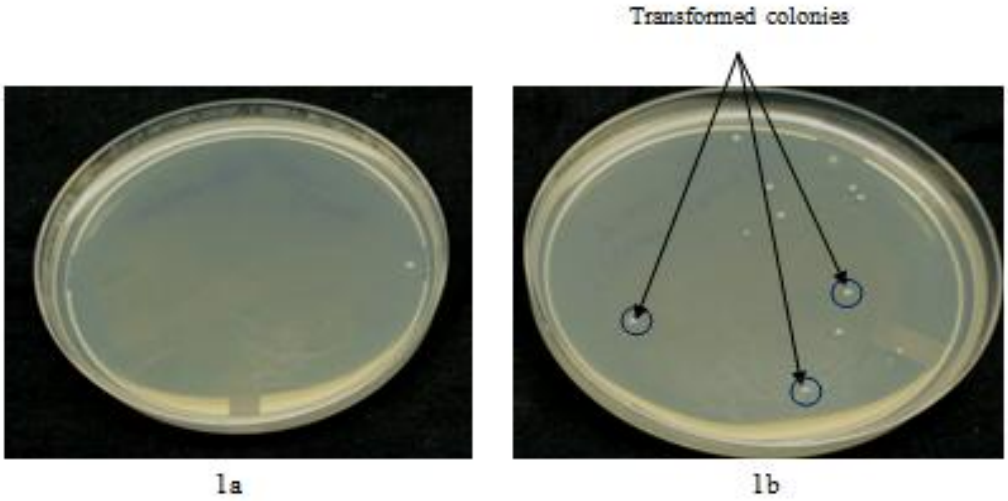


Plate 1. a. Control plate with no colonies b. Transformed plate with positive colonies

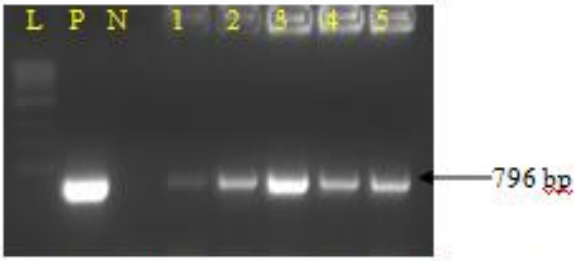


Plate 2. PCRanalysis of the plasmid DNA isolated from 5 randomly selected colonies L: 1 kb ladder, P: Positive control, N: Negative control, 1-5: PCR amplification of plasmid DNA showing presence of 796 bp *PDH47* gene

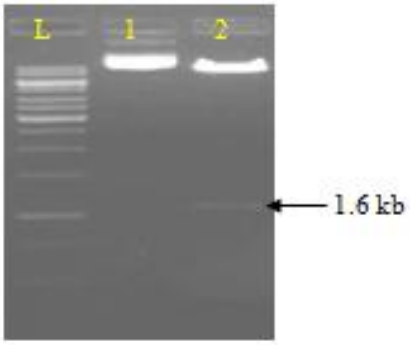


Plate 3. Restriction digestion of plasmid extracted from positive colonies L: 1 kb ladder, 1: undigested plasmid, 2: digested plasmid with *Nco*I (1.6 Kb)

p1301-PDH47 using immature embryos as explants. During co-cultivation of immature embryos with the *Agrobacterium* strain LBA4404 for 7 days, the green shoots developed from the germinated immature embryos were removed before transferring it on the resting medium (Plate 4a). In order to completely remove *Agrobacterium* after co-cultivation, the proliferated calli were kept in resting medium twice containing 250 mg/L of cefotaxime and 150 mg/L of Timentin at 10 days interval (Plate 4b and 4c). However, several reports suggested that the antibiotic cefotaxime can alone be used at a concentration of 250-500 mg/L for complete removal of *Agrobacterium* during washing and selection of the transformed rice calli (Shivachandran and Kalarani, 2009; Sarangi *et al.,* 2009; Sahoo *et al.,* 2011; Sikdar *et al*., 2015; Tran *et al*., 2015). After 10 days of culture in the second resting medium, the proliferated calli were divided into 4-6 smaller pieces (according to the size of the calli) and transferred to selection medium (CCMCH30) (composition shown in Appendix VIII) containing 30 mg/L Hygromycin B for three rounds of selection at 10 days of interval (Plate 4d, 4e and 4f). The healthy survived calli were transferred on to regeneration medium (RNMH30) containing 30 mg/L hygromycin B for 10 days and the calli developed green embryoids within 10 days of culture (Plate 4g and 4h) under continuous illumination at 5000lx at 28°C. The regenerated shoots developed roots within 15 days of culture when transferred on rooting medium (half strength MS medium without growth hormones) (Plate 4i and 4j). 24 putative transgenic plants of IR64 were regenerated, however only 19 putative transgenic lines successfully established in the transgenic green house with a regeneration efficiency of 3.59% (Table 4) (Plate 4k). Unfortunately, no transgenic plants could be regenerated in case of the Ranjit cultivar. Shivachandran and Kalarani, (2009) reported that the *indica* rice cultivar ASD16 lines regenerated on regeneration medium with a regeneration efficiency of 1.1% using scutellum derived embryogenic calli mediated through Agrobacterium method of genetic transformation. Likewise, Sahoo *et al*., (2011) also reported that the regeneration frequency ranged from 45-59% in *indica* rice cultivars such as IR64, CSR10, PB1 and Swarna using mature seeds as explants through *Agrobacterium* mediated method. Moreover, Nandakumar *et al*., (2007) reported that the regeneration efficiency ranged from 2.0 to 7.6% in *indica* rice cultivars using mature seeds derived calli mediated through *Agrobacterium* mediated gene transfer method.

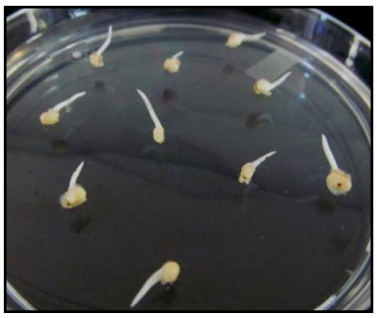
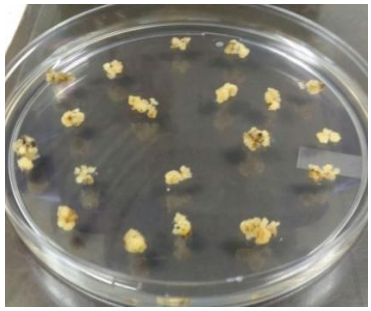
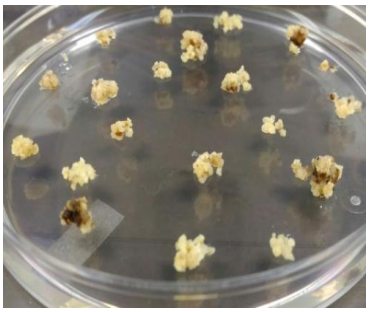


Plate 4a. Co-cultivation of immature embryos with *Agrobacterium* LBA4404 strain harbouring *p1301-PDH47* for 7days of culture

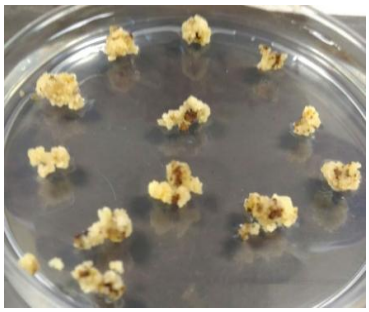
4b. Immature embryos in first resting medium containing 250 mg/L Cefotaxime and 150 mg/L Timentine for 10 days



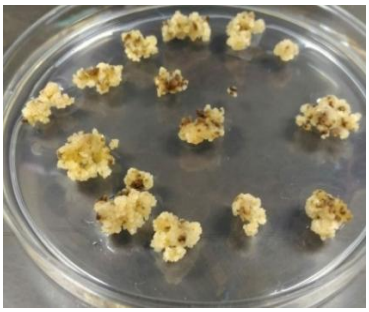
4c. Immature embryos in second resting medium containing 250 mg/L Cefotaxime and 150 mg/L Timentine for 10 days



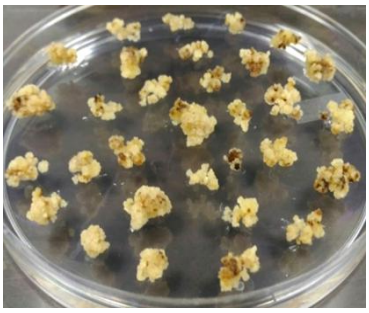
4d. Proliferated calli in 1st selection medium containing 30 mg/L Hygromycin B after 10 days of culture



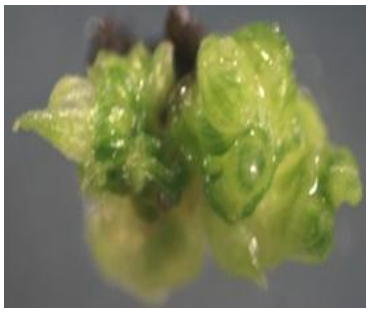
4e. Proliferated calli in 2nd selection medium containing 30 mg/L Hygromycin B after 10 days of culture



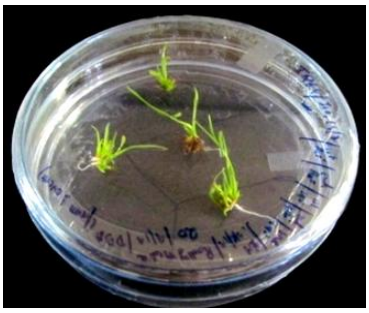
4f. Proliferated calli in 3rd selection medium containing 30 mg/L Hygromycin B after 10 days of culture



4g. Proliferated calli on CC regeneration medium after 10 days of culture



4h. Green embryoid formation on regeneration medium after 10 days of culture



4i. Rooting of *in vitro* regenerated shoots after 15 days of culture

4j. Putative transgenic plants with good root and shoot

4k. Established putative transgenic lines in the green house

Plate 4. *Agrobacterium* mediated genetic transformation using immature embryo with *PDH47* gene

Table 4. ***Agrobacterium*** **mediated genetic transformation of** ***indica*** **rice cv. Ranjit and IR64 using immature embryos as explants**

Rice cultivar	No. of embryos inoculated	No. of immature embryos survived in resting medium	No. of calli survived after			No. of lines regenerated	Regeneration efficiency (%)	No. of lines established in the green house	No. of PCR positive transgenic lines with *PDH47* transgene	Transformation efficiency (%)
			1st selection	2nd selection	3rd selection					
IR64	528	366	201	132	89	24	3.59	19	11	2.08
Ranjit	240	102	69	24	6	Nil	Nil	Nil	Nil	Nil

Regeneration efficiency (%) = No. of lines established/ No. of explants inoculated

Transformation efficiency (%) = No. of PCR positive plants/ No. of explants inoculated

4.3. Molecular analysis for the transgenic plants

4.3.1. Analysis of putative T_0 transgenic plants

4.3.1.1. PCR analysis

Genomic DNA was extracted from a total of 19 putative transformamts established in the green house as mentioned in the section 3.5.1 of previous chapter. Genomic DNA was subjected to PCR analysis using *PDH47* gene specific primers shown in (Table 2). PCR is a rapid technique to confirm the presence of transgene in the transgenic lines using gene specific primer that can generate a transgene specific product (Kohli *et al*., 2010). In the current study, out of 19 putative transgenic lines only 11 lines showed PCR positive as evident by presence of 796 bp of *PDH47* (Plate 5a and 5b) gene and 663 bp of *hptII* gene (Plate 6). In the current study, some of the T_0 transgenic lines showed negative PCR result for both *PDH47* and *hptII* genes. This might be due to transformed calli escaped the selection of Hygromycin B at the concentration of 30 mg/L. Bibi *et al.*, (2013) increased the dose of Hygromycin B from 50 mg/L to 75 mg/L to eliminate 80-85% of non-transformed plants in cotton. Moreover, Janna *et al*., (2000) reported that hygromycin was found to be an effective selective agent used in Dendrobium plant transformation as it was capable to cause complete death of the tissues at very low concentrations, *i.e.*, 10, 20 and 25 mg/L. Similarly, Htwe *et al*., (2014) reported that the hygromycin concentration 45 mg/L was capable of complete fatality of the tissue of the Malaysian rice line MR219. Thus, the hygromycin might be an effective selectable agent for identification of transformants in rice transformation. Nyaboga *et al*., (2014) also reported that the dose of the selective antibiotic is very crucial to minimize the selection of false positive plants in genetic transformation of any crop. Suboptimal dose of antibiotics results in high frequency of escapes (Datta *et al*., 1990). On the other hand, higher dose of antibiotic in selection medium will not only reduce the number false positive plants but also inhibit the growth of transformed cells thereby delay the process of regeneration (Wilmink and Dons, 1993). Suboptimal dose of antibiotics results in high frequency of escapes (Datta *et al*., 1990). Rosellini *et al*., (2007) reported that in tissue culture process the antibiotic degradation in the vicinity of transformed cells is a common phenomenon and might be responsible for inactivation of antibiotics. Plant susceptibility to antibiotics change broadly among species, genotypes and plant tissues (Padilla and Burgos, 2010). Excessively high antibiotic concentration may kill non-transformed cells; thereby

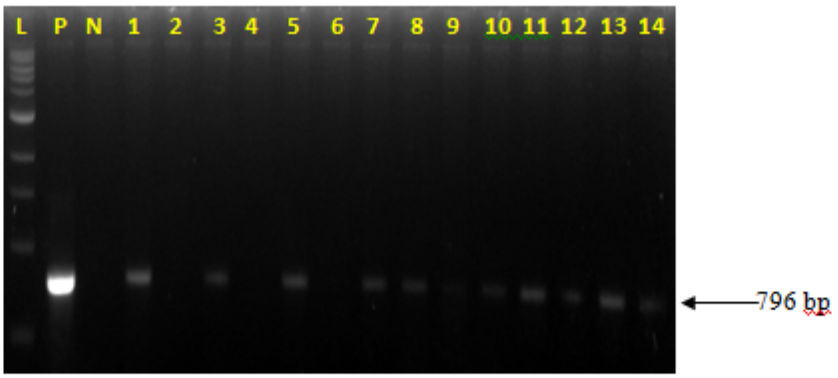


Plate 5a. PCR analysis of putative T_0 transgenic lines of IR64 for presence of *PDH47* transgene using gene specific primers. Lane L: 1 kb ladder, P: Positive control (*p1301-PDH47* plasmid), N: Negative control, 1: IR64 - 1/1 (+); 2: IR64 – 2/1 ; 3: IR64 – 3/1 (+); 4: IR64 – 4/1 ; 5: IR64 – 5/1(+); 6: IR64 -6/1 ; 7: IR64 – 7/1(+) ; 8: IR64 - 8/1 (+) ; 9: IR64 – 9/1 ; 10: IR64 – 10/1; 11: IR64 – 11/1(+); 12: IR64-12/1(+);13: IR64-13/1(+);IR64 – 14/1 (+ : PCR positive)

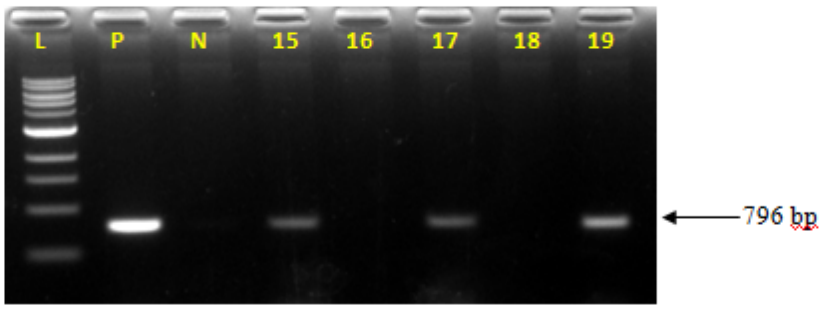
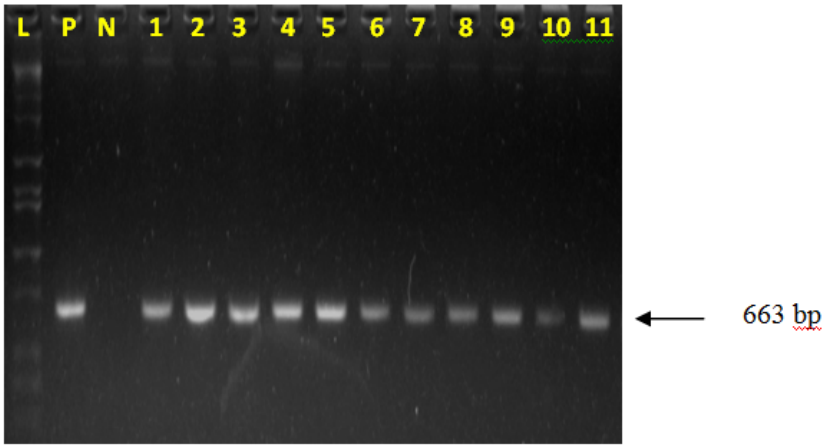


Plate 5b. PCR analysis of putative T_0 transgenic lines of IR64 for presence of *PDH47* transgene using gene specific primers. Lane L: 1 kb ladder, P: Positive control (*p1301-PDH47* plasmid), N: Negative control, 15: IR64 - 15/1 (+); 16: IR64 – 16/1 (-); 17: IR64 – 17/1 (+); 18: IR64 – 18/1 ; 19: IR64 – 19/1(+); (+ : PCR positive).

Plate 5: PCR analysis of putative T_0 transgenic lines of IR 64 for the presence of *PDH47* gene using gene specific primers



6. PCR analysis of 11 T_0 transgenic positive lines of IR64 for presence of *hptII* transgene using gene specific primers. Lane L: 1 kb ladder, P: Positive control (*p1301-PDH47* plasmid), N: Negative control, 1: IR64 – 1/1(+ ve), 2: IR64 – 2/1(+ ve); 3: IR64 – 3/1(+ ve) ; 4: IR64 – 4/1 (+ ve); 5: IR64 – 5/1(+ ve); 6: IR64 – 6/1(+ ve) ; 7: IR64 – 7/1(+ ve); 8: IR64 – 8/1(+ ve); 9: IR64 – 9/1(+ ve); 10: IR64 – 10/1(+ ve) ; 11: IR64 - 11/1(+ ve) ; (+ : PCR positive)

Plate 6: PCR analysis of putative T_0 transgenic lines of IR-64 for the presence of *hptII* gene using gene specific primers

inhibiting regeneration of transformed cells (Escandon and Hahne, 1991; Wilmink and Dons, 1993). In contrast, insufficient level of antibiotics may results in occurrence of many escapes and chimeras, thus inhibiting the regeneration and effective selection of transformed cells.

4.4. Relative expression level analysis of *PDH47* gene is T_3 transgenic lines through quantitative real time PCR (qRT-PCR)

Analysis of gene expression and identification of the candidate genes that involved in drought stress is important because it often projected as a solution for protecting crop plants against water scarce condition and increasing crop yield worldwide (Nelson *et al.,* 2007). In the present study, for gene expression analysis three T_3 homozygous transgenic lines (namely ASD16-46/1, ASD16-66/1 and ASD16-68/1) previously developed in the Department of Agricultural Biotechnology were used. Total RNA (Plate 8) was extracted from the leaf and root tissue of all the three homozygous lines using Trizol® reagent before and 18 days after drought stress (Plate 7a and 7b). cDNA was prepared from each sets of RNA sample as mentioned in the section 3.7 of the previous chapter. *Actin* gene was used as endogenous control gene in the real time PCR analysis. In all the three homozygous transgenic lines the expression of *PDH47* transcript found to be significantly more in the root tissue as compared to the leaf tissue (Fig. 4.1a, 4.1b and 4.1c). Vashisht *et al*., (2005) also reported that the expression of the *PDH47* gene was high in roots as compared to the shoots, indicating a role for *PDH47* in both the ABA-independent and ABA-dependent pathways in abiotic stress conditions. The higher level of expression of *PDH47* gene in root as compared to leaf tissues could be due to its association with the ABA signalling pathway which is predominantly occurs in the root tissue (Vashisht *et al.*, 2005). A very important part of drought sensing by roots is regulated by plant hormones and the predominant role is attributed to abscisic acid (ABA). ABA is synthesised in leaves and roots, and its production in roots is a well-known early response to water deficit (Schachtman and Goodger, 2008). ABA content increases very rapidly when cell turgor drops down and the elevation of ABA content was detectable just 30min after occurrence of stress (Xiong and Zhu, 2003). The process of ABA biosynthesis involves the epoxidation of zeaxanthin by zeaxanthin epoxidase (ZEP) that produces epoxyxanthophyll precursors (Taylor *et al.*, 2000). The elevated expression of the ZEP encoding gene was recorded in roots within 8 hrs of dehydration (Bray, 2002) indicating that ABA was actively

Nontransformed ASD16-46/1 ASD16-66/1 ASD16-68/1

Plate 7a: T_3 homozygous lines ASD16-46/1, ASD16-66/1 and ASD16-68/1 T_3 before drought stress (well watered condition)

Nontransformed ASD16-46/1 ASD16-66/1 ASD16-68/1

7b: T_3 homozygous lines ASD16-46/1, ASD16-66/1 and ASD16-68/1 after 18 days of drought stress

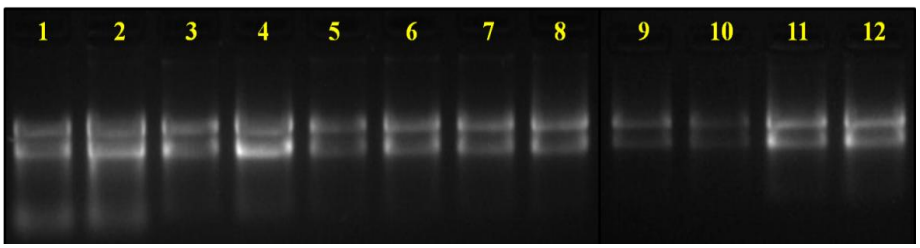


Plate 8: Total RNA extracted from the leaf and root tissue of T_3 homozygous lines (ASD16-46/1, ASD16-66/1 and ASD16-68/1). 1-2: ASD16-46/1 leaf, 3-4: ASD16-46/1 root, 5-6: ASD16-66/1 leaf, 7-8: ASD16-66/1 roots, 9-10: ASD16-68/1 leaf, 11-12: ASD16-68/1 root

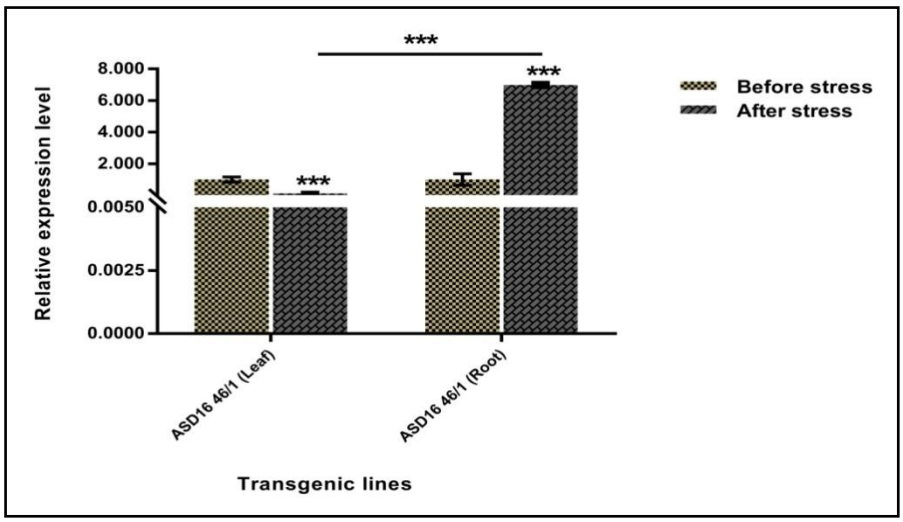
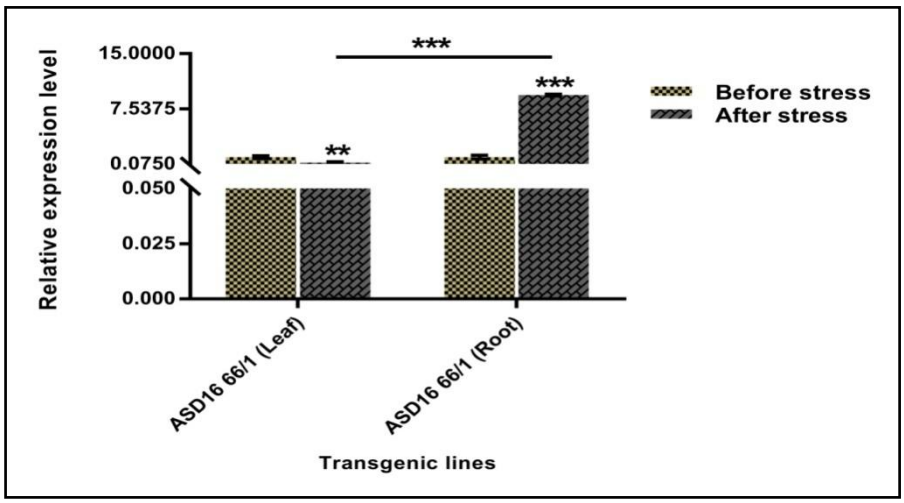
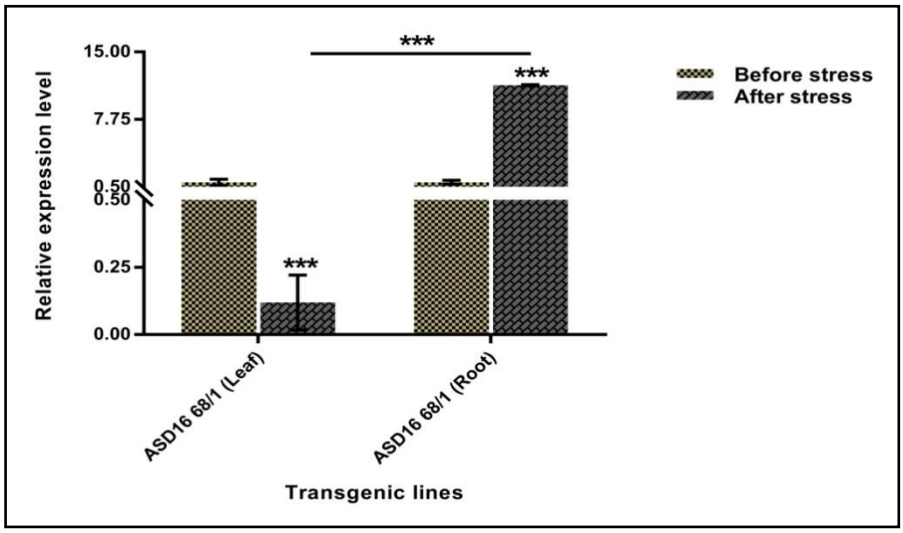


Fig. 4.1a: Relative expression of *PDH 47* transcript in leaf and root tissue of T_3 homozygous ASD16 46/1 line (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05 =NS)



4.1b: Relative expression of *PDH 47* transcript in leaf and root tissue of T_3 homozygous ASD16 66/1 line (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05 =NS)



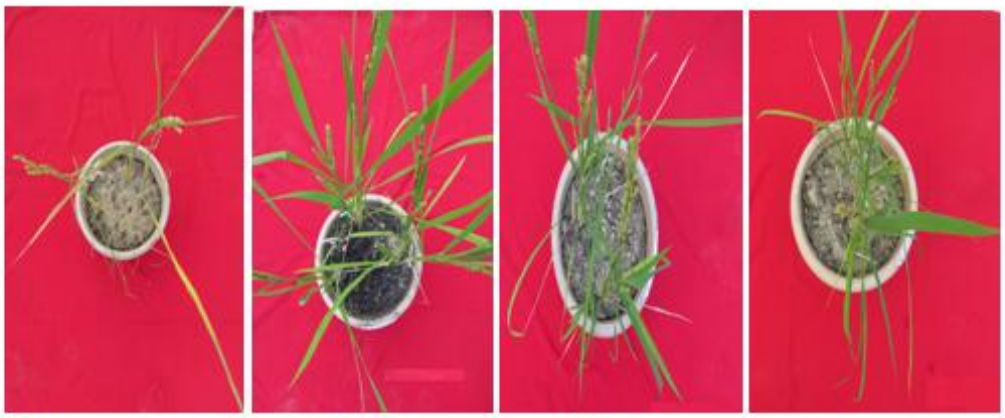
4.1c: Relative expression of *PDH 47* transcript in leaf and root tissue of T_3 homozygous ASD16 68/1 line (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)

synthesized in response to drought stress. Additionally, ABA concentration may be elevated by its release from conjugates in a reaction mediated by β-glucosidases (Dietz *et al.*, 2000; Wang *et al.*, 2011) and ABA conjugates may be the earliest resource of ABA before the set-up of its biosynthesis.

The most probable sites of ABA synthesis are root tips (Zhang and Tardieu, 1996) and the higher ABA content found in younger roots implicates that the capacity of ABA synthesis declines with root maturity (Puértolas *et al.*, 2014). The study of Shi and co-workers (2015) in rice showed that ABA accumulation in roots is important for early response to drought stress, but its high content in roots is not beneficial under prolonged and severe stress.

4.5. Physiological analyses of T_3 transgenic lines before and after drought stress in greenhouse conditions

Drought stress imparts significant negative impacts in almost all the cultivated crops at various stages of growth. In the current study, the 75 days old T_3 homozygous lines harbouring *PDH47* gene were subjected to drought stress for a period of 18 days following the procedure of Rahman *et al.*, (2016). The experiment was conducted in transgenic green house, Assam Agricultural University, Jorhat, to evaluate the growth performance of the transgenic rice lines over expressing *PDH47* gene under drought stress. Three 75 days old T_3 transgenic rice lines (ASD16-46/1, ASD16-66/1 and ASD16-68/1) were used in the current study along with nontransformed control rice plants. Two significant physiological parameters such as leaf wilting and leaf rolling were observed in the nontransformed rice plants when subjected to drought stress (Plate 9 and 10). All the physiological and biochemical analyses data were recorded before and after drought stress treatment. The T_3 transgenic rice lines showed delayed leaf wilting and leaf rolling even after drought stress as compared to the nontransformed control plants which clearly indicated that the expression of *PDH47* imparts drought tolerance in these transgenic rice lines (Plate 9 and 10). Leaf rolling was used as a criterion for scoring drought tolerance in tall and semi dwarf rice cultivars (Loresto *et al*., 1976; Loresto and Chang, 1981). Moreover, leaf rolling is one of the drought avoidance mechanisms to prevent water deficits during drought stress as the leaf rolling is induced by loss of turgor and poor osmotic adjustment (O'Toole and Chang, 1978; Hsiao *et al*., 1984). Wilting of leaf was observed when water potential of leaves and the osmotic potential of petiole cells were reduced under drought stress (Zhang *et al*.,2010).



Non-transformed ASD16-46/1 ASD16-66/1 ASD16-68/1

Plate 9: Leaf wilting in non-transformed control plant after drought stress. T_3 homozygous transgenic lines viz. ASD16-46/1, ASD16-66/1 and ASD16-68/1 did not show leaf wilting symptom.

Non-transformed ASD16-46/1 ASD16-66/1 ASD16-68/1

Plate 10: Leaf rolling in non-transformed control plant after drought stress. T_3 homozygous transgenic lines viz. ASD16-46/1, ASD16-66/1 and ASD16-68/1 showed no leaf rolling

Similarly, Dansana *et al*., (2014) also observed delayed leaf rolling in transgenic *indica* rice lines of Pusa Basmati 1 variety when transformed with *OsiSAP1* gene. Likewise, Abdula *et al.,* (2016) reported lower degree of leaf rolling in transgenic rice lines expressing *BrUGE1* transgene compared to their non-transgenic counterparts. The phenomenon of drought-induced drooping of leaves might have multiple physiological significance like reduction of both water loss from the leaf and the amount of light incident on the leaf surface, the later lowering the energy load on a leaf (Begg, 1980; Bjorkman and Demming-Adams, 1994).

4.5.1. Effect on chlorophyll content

Leaf chlorophyll content has a positive relationship with the rate of photosynthesis (Hotta *et al*., 1997). Reduction in chlorophyll content under any stress is a typical symptom of oxidative stress which results in photo-oxidation of pigments and chlorophyll degradation (Farooq *et al*., 2009). In the present study, the chlorophyll content of all the three transgenic rice lines *i.e.* ASD16-46/1, ASD16-66/1 and ASD16-68/1 were found to be more after drought stress as compared to the nontransformed control plant.

Before drought stress treatment, the total chlorophyll content of both the transgenic rice lines and the nontransformed control plant was almost similar, ranging from 0.8 to 1.1 (mg/g). But, after imposing to drought stress treatment, the total chlorophyll, chlorophyll a and chlorophyll b values of the nontransformed rice plants significantly decreased, whereas the total chlorophyll, chlorophyll a and chlorophyll b content of the transgenic rice lines showed no significance difference (Fig. 4.2a, 4.2b and 4.2c). Similarly, Latha *et al*., (2019) reported that drought stress significantly decreased total chlorophyll, chlorophyll a and chlorophyll b content in the nontransformed rice plants as compared to that of transgenic rice lines. Likewise, Sunitha *et al*., (2017) reported that transgenic *indica* rice cv. Samba Mahsuri (BPT 5204) overexpressing *Cajanus cajan* cold and drought regulatory protein encoding gene (*CcCDR*) showed higher chlorophyll content as compared to wild type plants under drought, salt and cold stress conditions. Transgenic rice and Arabidopsis expressing pigeonpea *CcHyPRP* gene could maintain higher chlorophyll contents when grown under different abiotic stress conditions (Priyanka *et al*., 2010a,b; Mellacheruvu *et al*., 2016). Moreover, Shivakumara *et al*., (2017) reported that overexpression of *PDH45* transgene imparts tolerance to multiple abiotic stresses in chilli by maintaining higher

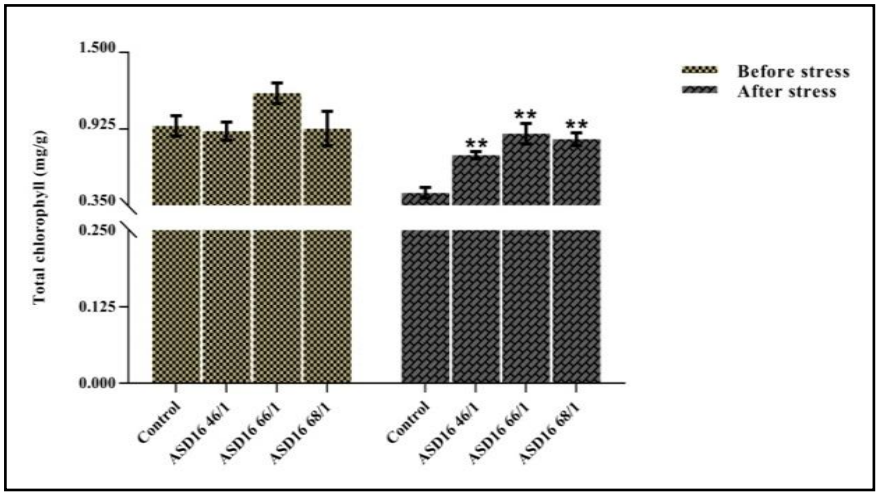
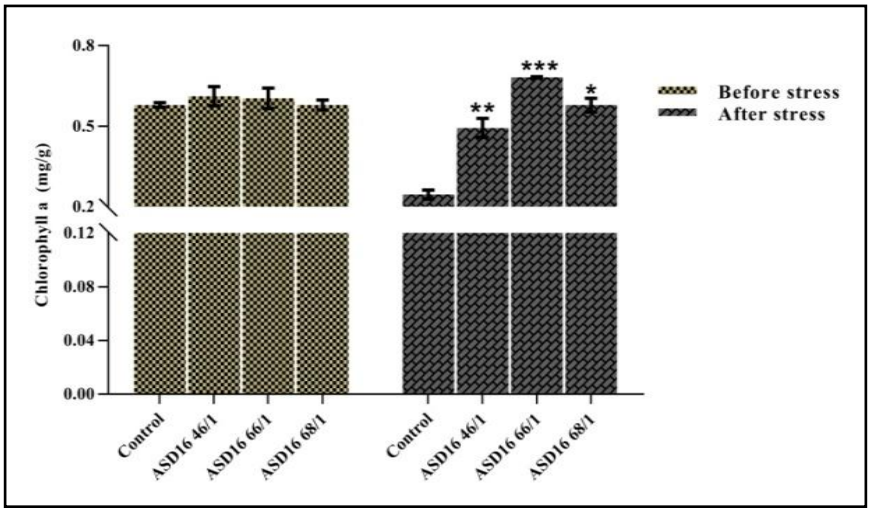
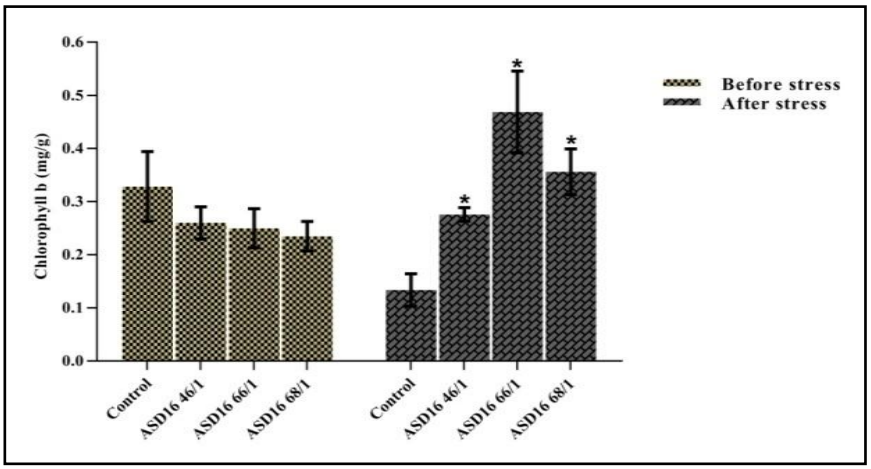


Fig. 4.2a: Total chlorophyll content (mg/g) of ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)



4.2b: Chlorophyll a content (mg/g) of ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)



4.2c: Chlorophyll a content (mg/g) of ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)

chlorophyll contents as compared to nontransformed plants. Sahoo *et al*., (2012) reported that the total chlorophyll content was found to be more in transgenic IR64 rice lines harbouring *PDH45* transgene under salt stress conditions. El-Esawi and Alayafi (2019) also found relatively higher total chlorophyll, chlorophyll a and chlorophyll b content in the transgenic rice lines harbouring the *OsRab7* gene under drought and salinity stress as compared to the non-transgenic counterparts. Our finding is also in agreement with Chen and co-worker (2019), where the authors demonstrated that the transgenic rice lines overexpressing *OsNAR2.1* gene showed significantly higher total chlorophyll, chlorophyll a and chlorophyll b content as compared to nontransgenic control plants.

4.5.2 Effect on leaf relative water content (RWC)

Relative Water Content (RWC) is considered to be a measurement of plant water status, reflecting the metabolic activity in leaf tissues and is used as the most meaningful index of water stress tolerance (Nayyar and Gupta, 2006). In the current study, the RWC of leaf of both transgenic rice lines and nontransformed rice plants was estimated to understand the physiological advantages imparted in the transgenic lines due to expression of *PDH47* transgene. Before drought stress treatment, there was no significant differences in the leaf RWC between control nontransformed rice plants and transgenic rice lines and it was within the range of 82% – 94% (Fig. 4.3). But after 18 days of drought stress, RWC was significantly decreased (62%) in the non-transformed rice plants as compared to transgenic rice lines (88%) (Fig. 4.3). All the transgenic rice lines *viz.* ASD16-46/1, ASD16-66/1 and ASD16-68/1 showed higher leaf RWC with percentages of 85.633%, 81.92% and 88.197%, respectively (Fig. 4.3). The RWC is often used to determine the water rentention capacity and drought tolerance, reflects the metabolic activities of plant tissues (Ravikumar *et al.,* 2014). Several studies suggested that enhanced leaf RWC during drought stress in transgenic plant expressing different drought responsive transgenes (Pardo, 2010; Datta *et al.,* 2012; Seth *et al.,* 2016). The reason for maintaining significantly high leaf RWC by the transgenic lines as compared to the nontransformed rice plants during drought stress might be not only due to stomatal closure (Datta *et al.,* 2012) but also by regulating stomatal density (Wang *et al.,* 2016).

4.5.3. Effect on water retention capacity (WRC)

The water retention capacity was used to determine the amount of water retained by the plants for maintenance of normal growth and development of the plants under any abiotic stress conditions. Water retention capacity (WRC) of both the transgenic lines and non-transformed rice plants was measured before and after drought stress following the methods mentioned in the section 3.7.3 of previous chapter. The WRC of all the three transgenic lines was found to be significantly higher (with value ranges from 101 to 126%) as compared to the non-transformed rice plants (with value 72.40%) (Fig. 4.4) after drought stress. However, no significant difference was observed in the WRC between the non-transformed rice plants and transgenic rice lines before drought stress. Transgenic peanut lines containing *AtDREB1A* exhibited improved water retention capacity as compared to water stressed control peanut lines, which could be due to the regulation of stomatal behaviour and transpiration under drought stress (Datta *et al.,* 2012; Anbazhagan *et al.,* 2015). Campo *et al.,* (2014) reported that over expression of a Calcium-Dependent Kinase of rice in *japonica* rice cv. Nipponbare exhibited higher water retention capacity under salt and drought stress**.** Likewise, the high water retention ability in the transgenic *Nicotiana benthamiana* plants constitutively expressing *TaPUB1* transgene from wheat might partially be a result of the increase in the contents of compatible osmolytes under drought stress conditions as reported by Zhang *et al*., (2017). Zhang *et al.,* (2011) also reported that expression of the harpin-encoding gene *hrf1* in rice increased the ABA content and water retention ability and enhanced drought tolerance. Transgenic rice lines expressing *mohrip1* and *mohrip2* genes from *Magnaporthe oryzae* showed improved water retention capacity under stress as compared to the non-transgenic control plants which might be due to regulation of ABA biosynthesis (Wang *et al.,* 2017). Our finding showed similar results as reported by Datta *et al.,* (2012), where the T_3 transgenic lines expressing the *PDH47* gene exhibited higher WRC as compared to that nontransformed rice plants under drought stress.

4.5.4. Determination of proline content

Proline is a common compatible osmoprotectant that accumulates in plants in response to different abiotic stresses. Proline accumulation is the first response of plants exposed to a drought stress condition in order to reduce cell injury. Zhu *et al*., (1998) reported increased in biomass content and higher accumulation of proline in

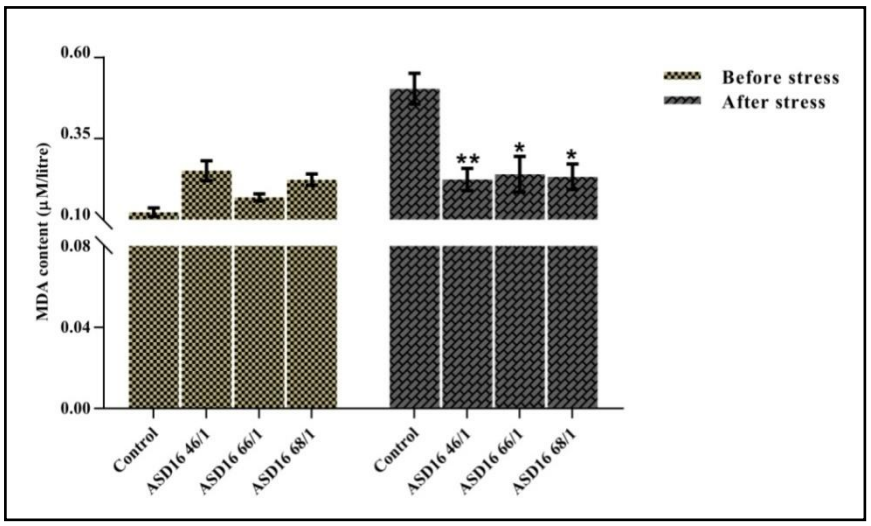


Fig. 4.3: Leaf RWC (%) of ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)

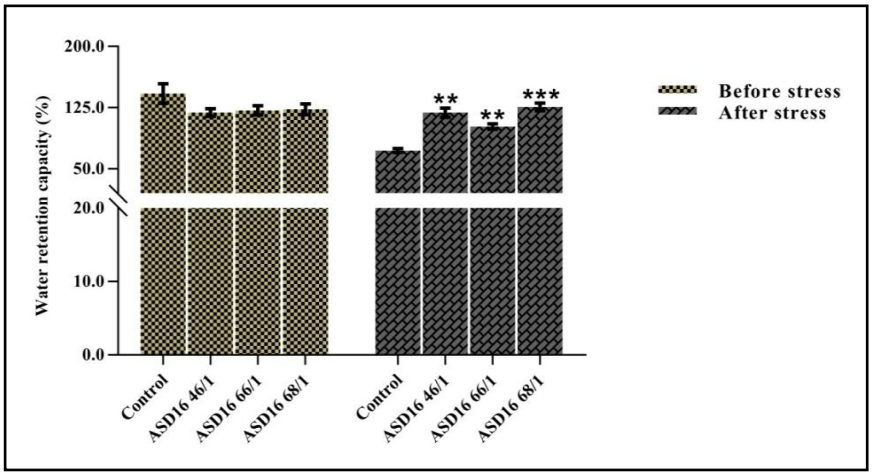


Fig. 4.4: WRC (%) of ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)

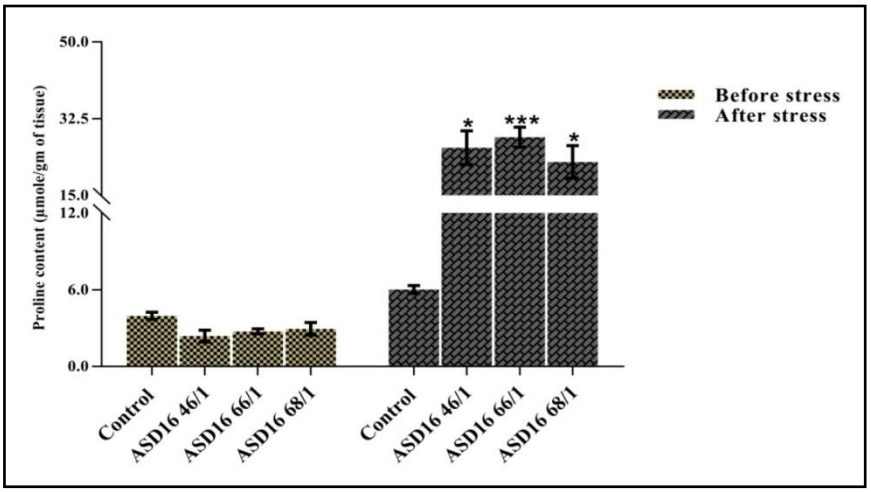


Fig. 4.5: Proline content (µmole/g of tissue) of ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)

transgenic plants under water-stress conditions as compared to non-transformed plants when pyrroline-5-carboxylate synthetase (*P5CS*) gene from mothbean (*Vigna aconitifolia* L.) was introduced into rice (*Orzya sativa* L.). Proline accumulation has been found to be correlated with drought and salt tolerance in plants (Delauney *et al*., 1993). Proline performs a diverse function in crop plants and acts as molecular chaperone, pH buffer, source of carbon and nitrogen during recovery and minimizes the harmful effect of ROS (Szabados *et al*., 2010). Proline has been reported to contribute approximately 10-15% of the osmotic adjustments in water stressed castor plants (Babita *et al.,* 2010), pea cultivars (Sánchez *et al.,* 1998) and transgenic sweet potato (Mbinda *et al*., 2018) under drought stress condition. In the present study, significantly higher accumulation of proline content was observed in all the three T_3 transgenic rice lines as compared to the non transformed rice plants after drought stress. The transgenic rice lines ASD16-46/1, ASD16-66/1 and ASD16-68/1 showed comparatively higher accumulation of proline content (29.231, 28.268 and 29.305 µmole/g of fresh tissue weight) as compared to nontransformed rice plants (6.021 µmole/g of fresh tissue weight) (Fig. 4.5). Transgenic rice plants expressing wheat *TaSTRG* gene exhibited higher proline and soluble sugar contents resulting in enhanced tolerance to multiple abiotic stresses (Zhou *et al.*, 2009). The transgenic rice overexpressing zinc finger protein (*ZFP245*) showed increased proline levels and elevated expression level of pyrroline-5 carboxylate synthetase gene under stress conditions which resulted in enhancd tolerance to cold, drought, and oxidative stress conditions (Huang *et al*., 2009). Likewise, Geda *et al*., (2019) found higher proline content in the transgenic *indica* rice cv. Pusa Basmati 1 expressing *AtDREB1A* gene under drought stress conditions. Ravikumar *et al.,* (2014) also found higher proline content in the transgenic *indica* rice lines of Samba Mashuri expressing *AtDREB1A* gene under drought stress as compared to the non-transformed control plants. Overexpression of the heat shock protein gene *OsHsp17.0,* or *OsHsp23.7,* has been found to improve tolerance to drought and salinity stresses in transgenic rice lines as compared to the non-transformed control plants due to increased accumulation of proline (Zou *et al.,* 2012). Tang *et al.,* (2019) demonstrated that increased drought and salinity stress tolerance in transgenic rice lines overexpressing *MYB* transcription factor was due to higher proline content.

4.5.5. Estimation of malondialdehyde (MDA) content

When ROS levels exceed the capacity of the plant to scavenge, lipid peroxidation (LP) in biological membranes increases, thereby affecting the

physiological processes of the cell. Malondialdehyde (MDA) is one of the final products of oxidative modification of lipids, and is responsible for cell membrane damage including changes to the intrinsic properties of the membrane, such as fluidity, ion transport, loss of enzyme activity and protein cross–linking. These changes eventually result in cell death (Sharma *et al.,* 2012; Sakhanokho *et al*., 2004 and Meloni *et al*., 2003). Lipid peroxidation was measured in terms of malondialdehyde (MDA) content, a product of lipid peroxidation and indicator of oxidative damage, caused by drought and salinity stresses in plants (Del Rio *et al.,* 2005). In the present study, MDA content of both transgenic and non-transformed rice plants was estimated before and after drought stress (Fig. 4.6). The transgenic lines namely ASD16 46/1, ASD16 66/1 and ASD16 68/1 after drought stress showed MDA content of about 0.2, 0.23 and 0.21 μM/litre respectively in contrast to the non-transformed control plant which showed 0.54 μM/litre (Fig. 4.6). However, before drought stress the transgenic lines and non-transformed control plant showed no significant difference in MDA content. It is possible that the detrimental effect of water deficit in soil and genotypic variation in drought tolerance is associated with levels of lipid peroxidation in tissues. Campo *et al.,* (2014) reported that transgenic rice plants overexpressing *OsCPK4* gene showed minimal MDA accumulation than control plants. Similarly, El-Esawi and Alayafi (2019) also reported significantly lower accumulation of MDA in the transgenic rice lines over-expressing the *OsRab7* gene under drought and salinity stress as compared to the non-transformed control plants. Tang *et al.,* (2019) showed that transgenic rice lines overexpressing *MYB* transcription factor had relatively less accumulation of MDA during drought stress as compared to their non-transgenic counterparts. Thus, *PDH47* over expression in transgenic rice plants might be counteracting the toxic ROS effects and reduce the oxidative damage, conferring greater tolerance to drought stress. Comparatively lower accumulation of MDA in transgenic plants in contrast to the non-transformed control plants has not only been reported in rice but also in some other crops. Singh *et al.,* (2012) showed that instability of biological membranes, as reflected by lipid peroxidation, was greater in drought–sensitive than in drought–tolerant wheat (*Triticum aestivum* L.) genotypes. Likewise, Sunitha *et al*., (2017) found lower MDA content in the transgenic *indica* rice Samba Mahsuri (BPT 5204) lines expressing *CcCDR* gene compared to the control plants under drought, salt and cold stresses.

4.5.6. Estimation of Electrolyte leakage (EL)

Electrolyte leakage is a hallmark of stress response in intact plant cells. This phenomenon is widely used as a test for the stress-induced injury of plant tissues and 'a measure' of plant stress tolerance (Levitt, 1972; Blum and Ebercon, 1981; Bajji *et al.,* 2002; Lee and Zhu, 2010). The electrolyte leakage is ubiquitous among different species, tissues and cell types and can be triggered by drought (Blum and Ebercon, 1981; Leopold *et al.,* 1981; Shcherbakova and Kacperska, 1983). Muzny *et al.,* (2006) reported that drought stress treatment leads to increase in leakage of ions by altering the structures of the membrane proteins. Huang *et al*., (1997) suggested that estimation of ion leakage would be an indirect way of measuring the drought stress tolerance ability in diverse plant species. Therefore, in the current study the EL of both T_3 transgenic rice lines and non-transformed rice plant was estimated. Before drought stress the EL values of both untransformed control rice plant and transgenic rice lines were almost similar, ranging from 6.8% to 13.23% (Fig. 4.7). After drought stress, the ion leakage of nontransformed rice plants rapidly increased to 42.2% in contrast to the three transgenic rice lines showed 26.9%, 24.53% and 23.83% of EL, respectively (Fig. 4.7). Wang *et al.,* (2005) reported that transgenic rice plants overexpressing the gene encoding manganese superoxide dismutase, an antioxidant enzyme, have lower EL than in non-transgenic plants under drought stress. Our finding is in agreement with Gao *et al*., (2013) where the authors reported that non-transformed rice plants under drought stress conditions showed severe leakage of ions as compared to the transgenic rice plants expressing *TaLEA* transgene. Transgenic rice plants overexpressing *OsSUV3* show reduced EL thereby leading to enhanced tolerance to drought and salinity stresses (Tuteja *et al.,* 2013). Similarly, Joshi *et al.,* (2020) reported that transgenic rice plants harbouring trehalose-6-phosphate synthase/phosphatase (TPSP) from *E. coli* have lower EL as compared to the non-transgenic counterparts under drought, saline and sodic conditions.

4.5.7. Estimation of Glycine Betaine content

The acclimation of a plant to a constantly changing environment involves the accumulation of certain organic compounds of low molecular mass, collectively known as compatible solutes, in the cytoplasm (Rathinasabapathi *et al.,* 2000; Chen and Murata, 2002). The evidence from numerous investigations of the physiology, genetics, biophysics and biochemistry of plants strongly suggests that

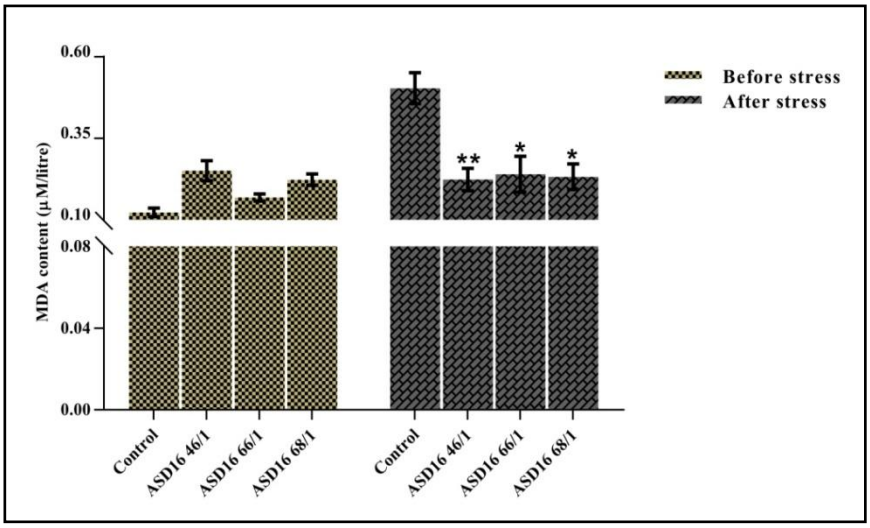


Fig. 4.6: MDA content (µM/L) of ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)

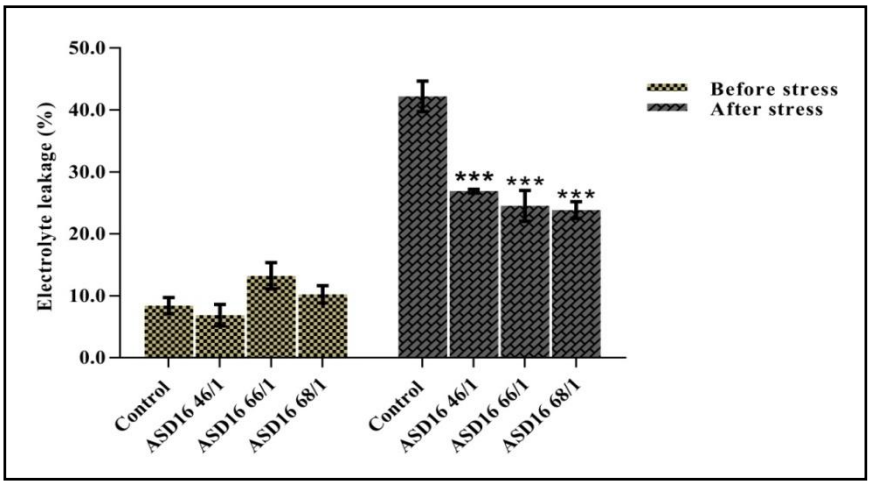


Fig. 4.7: Electrolyte leakage (%) of ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)

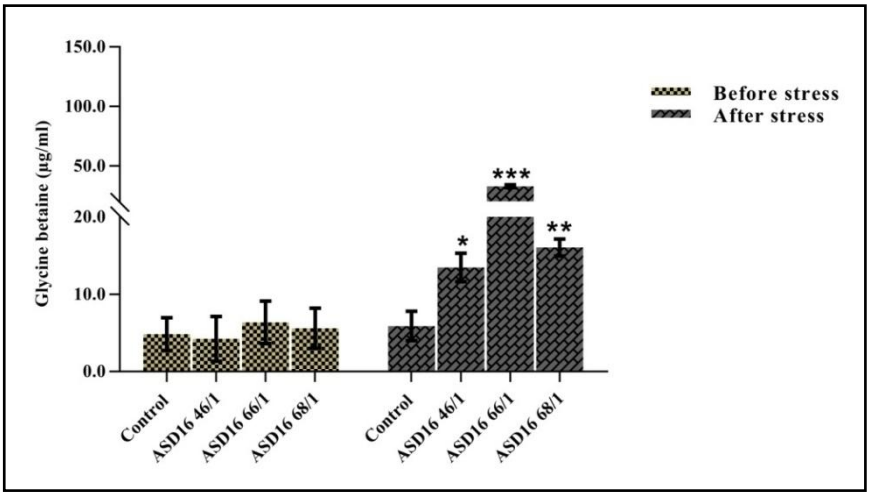


Fig. 4.8: Glycine betaine content (µg/ml) of ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)

glycine betaine (GB), an amphoteric quaternary amine, plays crucial role as a compatible solute in plants under various types of environmental stress, such as high levels of salts, low temperature and drought (Sakamoto and Murata, 2001; Bohnert *et al*., 1995). The effect of drought stress treatment in the quaternary ammonium compound glycine betaine was measured. In the current study, after 18 days of drought stress, the accumulation of glycine betaine was found to be significantly higher in the transgenic lines as compared to non-transformed rice plant (Fig. 4.8). However, no significant difference between non-transformed rice plants and transgenic lines were observed before drought stress. The introduction of *codA* gene transgenic rice lines resulted in increased accumulation of GB under stress as compared to non-transgenic plants (Alia and Murata, 1998; Kathuria *et al.,* 2009). The exits several reports suggested that higher accumulation of glycine betaine in plant species in response to drought stress (Ashraf and Foolad, 2007; Chen *et al.,* 2007; Mohanty *et al.,* 2002). Glycine betaine was found to be involved in inhibiting reactive oxygen species accumulation, protection of photosynthetic machinery, activation of some stress related genes and membrane protection (Chen and Murata, 2008). Glycine betaine has also been implicated in protection of quaternary structure of proteins by maintaining the enzyme activity from the damaging effects of environmental stresses (Sakamoto and Murata, 2002).

4.5.8. Histochemical detection of Reactive Oxygen Species (ROS)

One of the first and foremost responses of plants to drought stress is accumulation of reactive oxygen species (ROS) in different tissues (Cruz de Carvalho, 2008). These reactive oxygen species (ROS) mostly trigger the induction of defense mechanisms and acclimation responses which leads to the adaptation of plant metabolism to a changing environment. But in severe stress condition higher accumulation of ROS can cause damage of nucleic acid, protein, lipids and other cellular components (Mittler, 2002; Anjum *et al.,* 2011b, Sharma *et al*., 2012). In higher plants, the impacts of ROS production under drought stress condition is well documented (Rejeb *et al.,* 2014). In the present study, the accumulation of ROS in the tip portion of the leaf tissues of T_3 transgenic lines and non-transformed rice plants was recorded by DAB staining (Fig. 4.9). A marked increase in the brown coloured precipitate was observed in the leaf tissue of the non-transformed control rice plants as compared to that of T_3 transgenic lines (Fig. 4.9) after drought stress. Cellular membranes and macromolecules were better protected with less accumulation of ROS

in transgenic rice lines overexpressing *PDH47* transcripts as compared to non-transformed control plants which might be because of efficient ROS scavenging in transgenic rice lines. Several reports also showed similar pattern of ROS accumulation in the transgenic rice lines under drought stress conditions when transformed with drought responsive genes like *ZFP245* (Huang *et al.,* 2009), *AtWRKY57* (Jiang *et al.*, 2016), *AtDREB1A* (Ravikumar *et al.*, 2014).

4.5.9. Effect on net photosynthetic rate

Drought stress leads to a significant reduction in the rate of net photosynthesis because of closure of stomata, which restricts the CO_2 diffusion into the leaf (Osorio *et al*., 2011). In the present investigation, the net photosynthetic rate was significantly decreased in the non-transformed plants as compared to transgenic rice lines (ASD16-46/1, ASD16-66/1 and ASD16-68/1) during drought stress (Fig. 4.10). However, the net photosynthetic rate of both transgenic rice lines and non-transformed control rice plants showed no significant differences before drought stress (Fig. 4.10). Similar observation was reported by Geda *et al*., (2019), where the authors found that the net photosynthesis rate in transgenic *indica* rice cultivar Pusa Basmati 1 was higher under drought stress as compared to their non-transgenic counter parts due to over expression of *AtDREB1A* transfcription factor. Zhang *et al.,* (2017) also reported increased net photosynthetic rate in response to drought stress in transgenic rice lines expressing *TaPUB1* transgene as compared to the nontransformed control plants. Babitha *et al*., (2015) also reported that the transgenic tobacco plants overexpressing *EcbHLH57* showed higher photosynthetic rate under drought stress condition that resulted in higher biomass. Likewise, Augustine *et al.,* (2015) found higher photosynthetic rate in transgenic sugarcane plants expressing *PDH45* gene under drought and salinity stress conditions. Tuteja *et al*., (2013) also reported that the rice SUV3 transgenic lines showed higher net photosynthetic rate under salinity stress as compared with wild type plants. Garg *et al*., (2017) reported that net photosynthetic rate was significantly increased in *PDH45* and *PDH45 + EPSPS* overexpressing transgenic plants as compared to wild type plants under salinity stress.

4.5.10. Effect on stomatal conductance

In the current study, significant variation in the stomatal conductance was observed between the T_3 transgenic lines and non-transformed control plants after drought stress. T_3 transgenic lines showed higher stomatal conductance as compared to

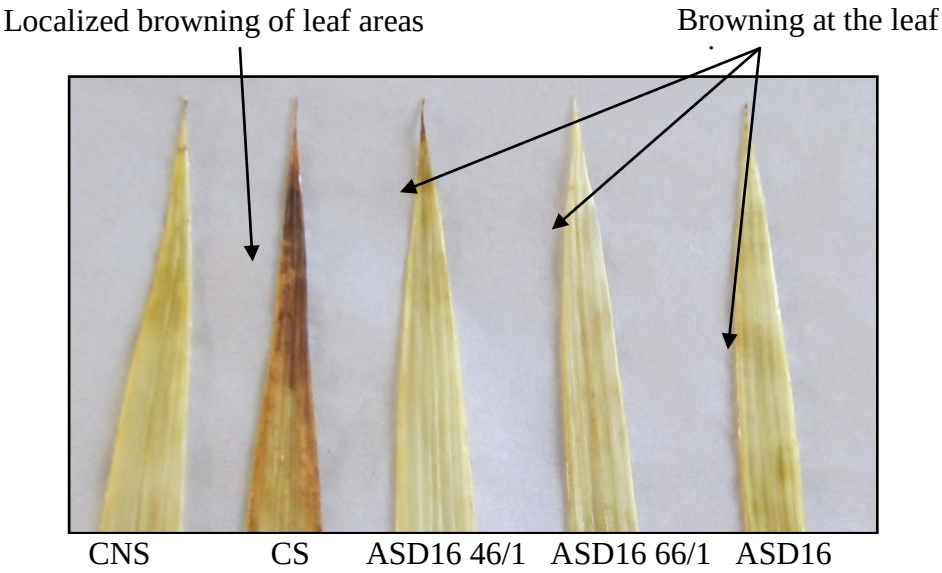


Fig. 4.9: Detection of ROS accumulation using DAB staining. CNS: Control non-stressed leaf, CS: Control stressed leaf, ASD16 46/1, ASD16 66/1 and ASD16 68/1 are transgenic lines after drought stress.

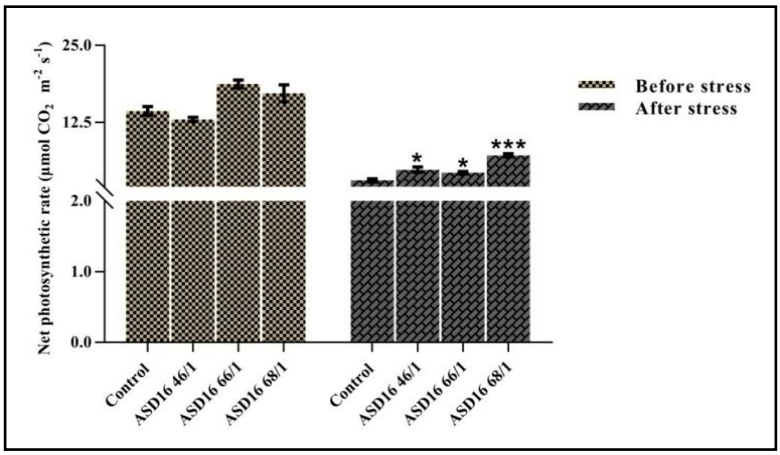


Fig. 4.10: Net photosynthesis rate (µmole CO_2 m^{-2} s^{-1}) of ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)

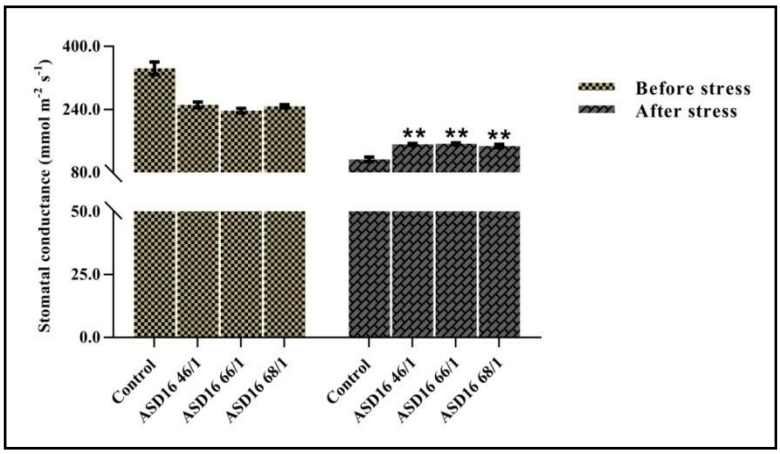


Fig. 4.11: Stomatal conductance (mmole m^{-2} s^{-1}) of ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)

non-transformed rice plants after drought stress. However, no significant difference was observed between the transgenic lines and non-transformed rice plants before drought stress (Fig. 4.11). It indicated that the CO_2 accumulation inside stroma of the chloroplast of transgenic rice lines was more in comparison to the non-transformed control plants under drought stress. Similarly, increased in stomatal conductance was observed in response to drought stress by Zhang *et al.,* (2017) in transgenic *Nicotiana benthamiana* plants expressing wheat *TaPUB1* gene. Geda *et al.*, (2019) also observed higher stomatal conductance in transgenic *indica* rice cultivar Pusa Basmati 1 under drought stress due to over expression of *AtDREB1A* transcription factor. Likewise, Babitha *et al*., (2015) also reported that the transgenic tobacco plants overexpressing *EcbHLH57* showed higher stomatal conductance under drought stress condition that resulted in higher biomass. Augustine *et al.,* (2015) also found the higher stomatal conductance in transgenic sugarcane plants expressing *PDH45* gene under drought and salinity stress conditions. Tuteja *et al*., (2013) also reported that the rice SUV3 transgenic lines showed higher stomatal conductance under salinity stress as compared with wild type plants. Garg *et al*., (2017) reported that stomatal conductance was significantly increased in *PDH45* and *PDH45 + EPSPS* overexpressing transgenic plants as compared to wild type plants under salinity stress.

4.5.11. Effect on transpiration rate

Gas exchange parameters are significantly affected by adverse effects of various abiotic stresses. To evaluate whether *PDH47* transgene expression could improve gas exchange parameters under drought stress, the transpiration rate of both the transgenic rice lines and non-transformed control plants was measured. Before drought stress the transgenic lines showed no significant difference in the transpiration rate with that of control non-transformed plants. Under drought stress condition, decrease in transpiration rate was observed in non-transformed rice plants. However, all the three transgenic lines revealed significantly higher transpiration rate as compared to the non-transformed rice plants after drought stress (Fig. 4.12), indicating that *PDH47* overexpressing rice lines exhibited greater tolerance to drought through enhancing the leaf RWC and other gas exchange characteristics. El-Esawi and Alayafi (2019) also reported significantly higher transpiration rate in the transgenic rice lines over-expressing the *OsRab7* gene under drought and salinity stress as compared to the non-transformed control plants. Similarly, Geda *et al*., (2019) also observed higher transpiration rate in transgenic *indica* rice cultivar Pusa Basmati 1 as compared to their

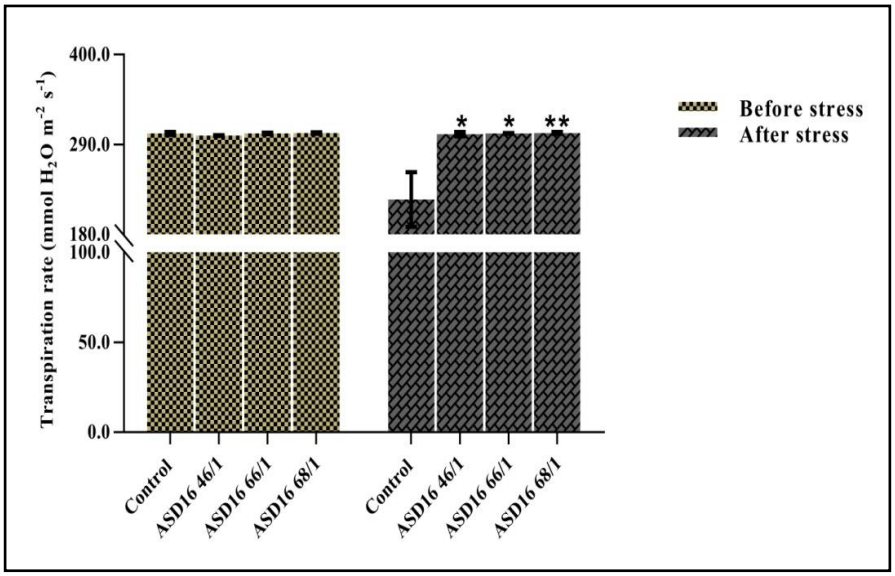


Fig. 4.12: Transpiration rate (mmole H_2O m^{-2} s^{-1}) of ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)

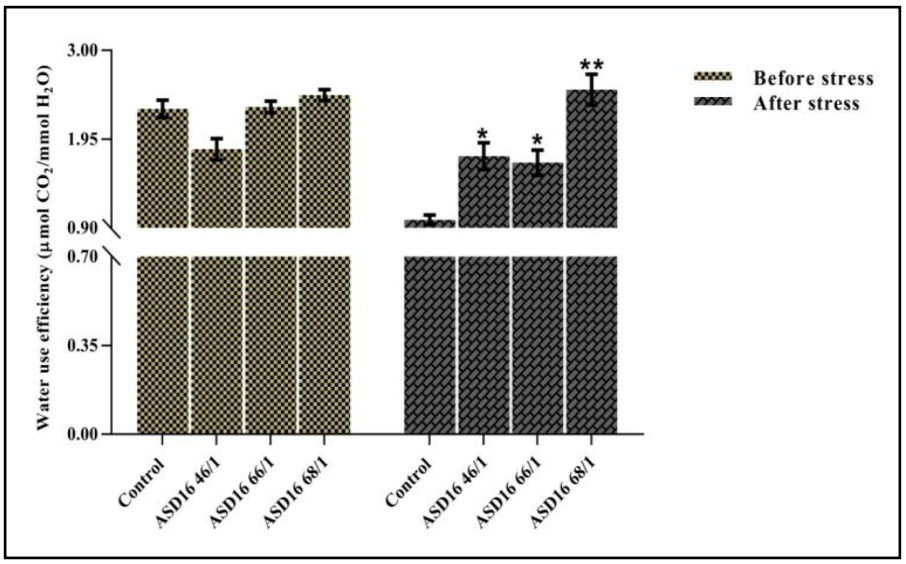


Fig. 4.13: WUE (µmole CO_2/mmole H_2O) of ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)

non-transgenic counterparts under drought stress due to over expression of *AtDREB1A* transcription factor. Augustine *et al.,* (2015) also found the higher transpiration rate in transgenic sugarcane plants expressing *PDH45* gene under drought and salinity stress conditions. Garg *et al*., (2017) reported that transpiration rate was significantly increased in *PDH45* and *PDH45* + *EPSPS* overexpressing transgenic plants as compared to wild type plants under salinity stress.

4.5.12. Effect on water use efficiency (WUE)

The WUE of both the transgenic lines and non-transformed rice plants was measured in the present study before and after drought stress. The transgenic lines showed no significant difference in WUE with that of non-transformed control rice plants before drought stress. However, under drought stress the non-transformed control rice plants showed decrease level of WUE whereas, all the three transgenic lines over-expressing the *PDH47* gene constantly maintained a significantly higher level of WUE (Fig. 4.13). Similarly, Geda *et al*., (2019) also observed higher WUE in transgenic *indica* rice cultivar Pusa Basmati 1 under drought stress due to over expression of *AtDREB1A* transcription factor. Banavath *et al.,* (2018) reported that transgenic peanut lines over-expressing *AtHDG11* gene exhibited significantly higher WUE under salt and drought stress conditions. Similarly, Karaba *et al*., (2007) reported that transgenic *japonica* rice cultivar Nipponbare overexpressing *HARDY* gene showed higher WUE under drought and salt stress conditions. Transgenic wheat constitutively expressing the barley *HVA1* gene improved water use efficiency under water deficit conditions as reported by Sivamani *et al*., (2000). Li *et al*., (2019) also reported that a leucine-rich repeat-receptor-like kinase gene *SbER2–1* from sorghum expressed in maize conferred increased drought tolerance by enhancing water-use efficiency. *PdERECTA*, a leucine-rich repeat receptor-like kinase of poplar confers enhanced water use efficiency in Arabidopsis as reported by Xing *et al.,* (2011). Guo *et al*. (2016) also observed that the dehydration-induced *ERECTA MsSIK1* genes from alfalfa improved water use efficiency in transgenic Arabidopsis.

4.5.13. Effect of drought stress on antioxidant enzyme activities

Antioxidative enzymes play a crucial role in scavenging ROS and improving plant tolerance to environmental stresses (El-Esawi *et al.,* 2018). Therefore, in the present study the activities of ascorbate peroxidase and catalase were estimated both in the non-transformed rice plant and T_3 transgenic lines overexpressing the *PDH47* gene before and after drought stress. Before commencement of the drought,

there were no significant difference in the antioxidative enzymes activities between the nontransformed rice plants and T_3 transgenic lines (Fig. 4.14a and 4.14b). However, after drought stress, remarkable increase in the antioxidative enzymes activities were observed in the transgenic lines as compared to non-transformed rice plants (Fig. 4.14a and 4.14b). The difference of antioxidative enzymes activities between the non-transformed rice plants and the transgenic lines was highly significant. Similar results were reported by El - Esawi and Alayafi (2019) where the authors found significant increased level of antioxidative enzymes activities like ascorbate peroxidase, catalase *etc.* in the transgenic rice lines over-expressing the *OsRab7* gene under drought and salinity stresses as compared to the non-transformed control plants. Zhang *et al.,* (2017) also demonstrated higher antioxidative enzymes (ascorbate peroxidase and catalase) activities in the transgenic plants of *N. Benthemiana* expressing the *TaPUB1* gene under drought stress condition. Similarly, Tuteja *et al*., (2013) reported that the rice SUV3 transgenic lines showed higher activities of antioxidant enzymes under salinity stress as compared with wild type plants. Garg *et al*., (2017) reported that the antioxidant enzymes were significantly increased in *PDH45* and *PDH45 + EPSPS* overexpressing transgenic plants as compared to wild type plants under salinity stress.

4.5.14. Effect of drought stress on root architecture system

The root architecture system of both the non-transformed rice plants and T_3 transgenic lines were studied before and after drought stress by growing the plants in the basket and PVC pipes (Fig. 4.15). All the root parameters such as root length, root number, root thickness, root dry biomass, shoot dry biomass and root to shoot ratio were studied and compared between transgenic lines and non-transformed rice plants before and after drought stress. In the current study, all root parameters were significantly higher in the transgenic lines as compared to the nontransformed rice plants after drought stress (Fig. 4.17a, 4.17b, 4.17c, 4.17d, 4.17e and 4.17f). However, the root parameters showed no significant difference both in transgenic lines and non-transformed rice plants before drought stress. However, all the root parameters of the transgenic lines such as root length, root number, root thickness, root dry biomass, shoot dry biomass and root to shoot ratio showed higher values over nontransformed control rice plants after drought stress (Fig. 4.17a, 4.17b, 4.17c, 4.17d, 4.17e and 4.17f), which might be due to over-expression of *PDH47* transgene in the root tissues as compared to leaf tissues. Among all root parameters, root length of the transgenic rice lines showed

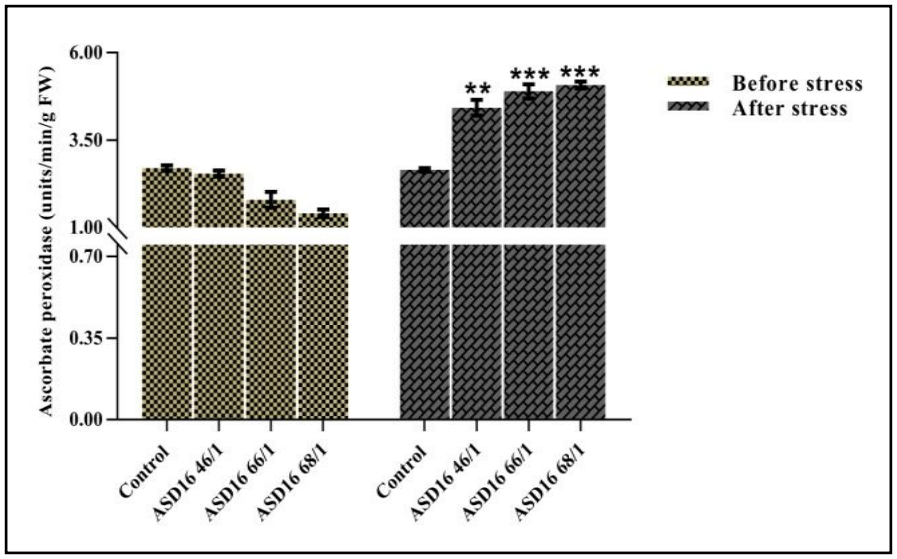


Fig. 4.14a: Ascorbate peroxidase activity (units/min/g FW) of ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress treatment (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)

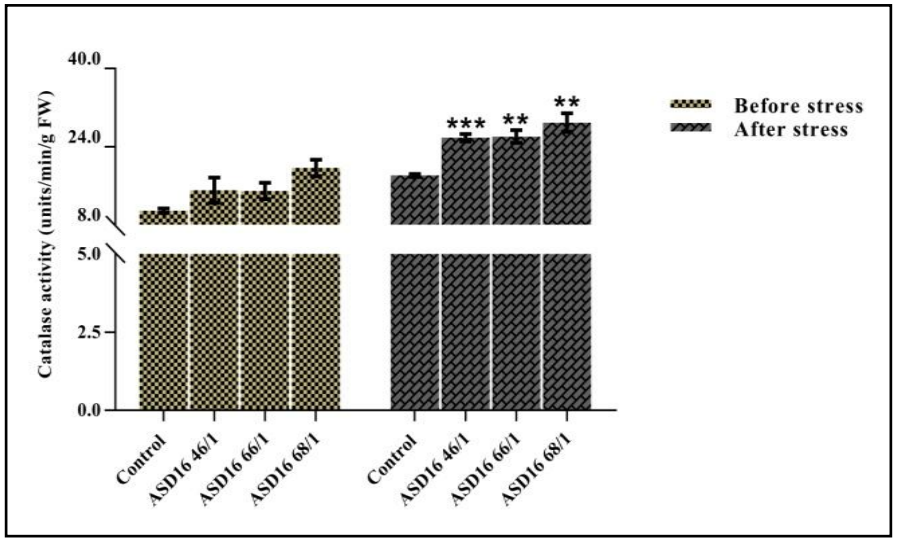


Fig. 4.14b: Catalase activity (units/min/g FW) of ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)

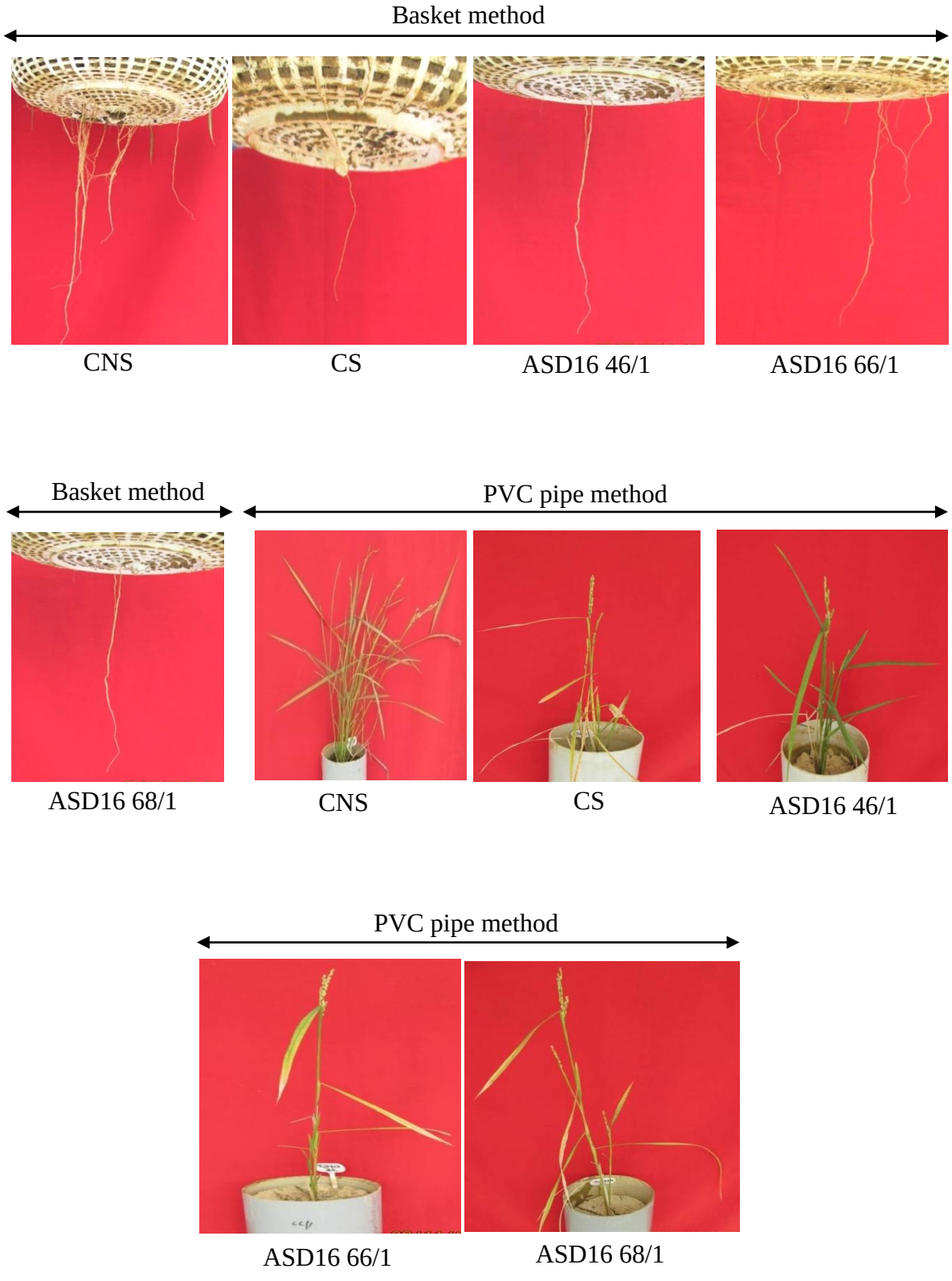


Fig. 4.15: Root length study of nontransformed control and transgenic lines before and after drought stress by basket and PVC pipe method. CNS: Control non-stressed, CS: Control stressed and ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines subjected to drought stress

Fig. 4.16: Root architecture study of nontransformed control and transgenic lines before and after drought stress.CNS: Control non-stressed, CS: Control stressed and ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines subjected to drought stress.

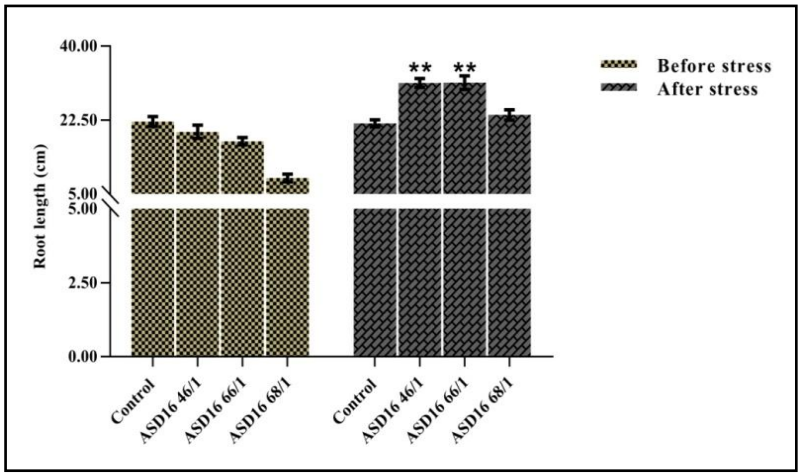
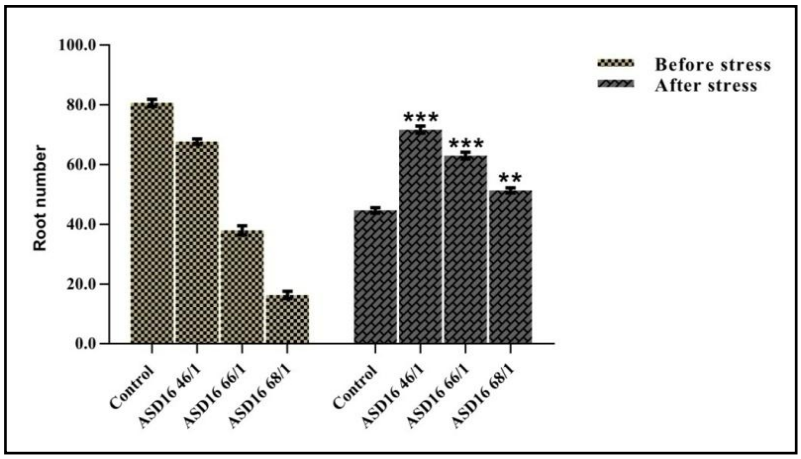
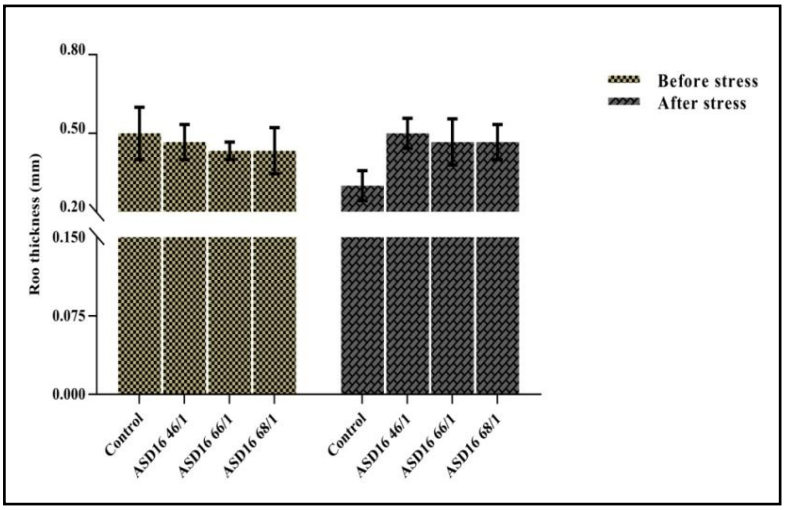


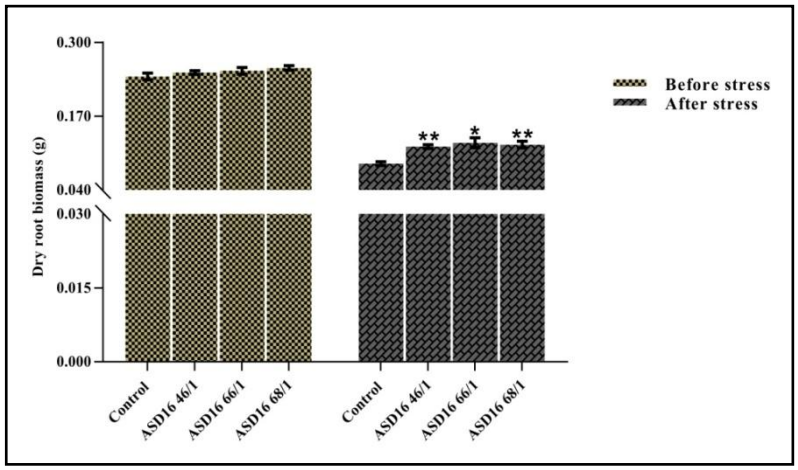
Fig. 4.17a: Study of root length (cm) of ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)



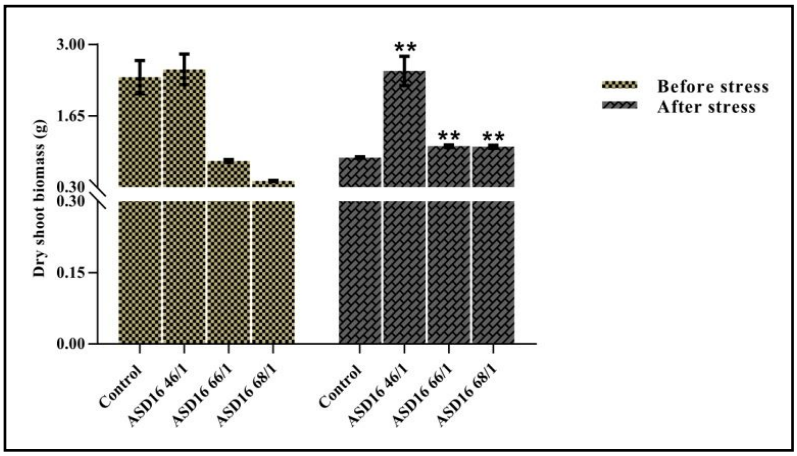
4.17b. Study root number of ASD16 46/1 , ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)



4.17c. Study of root thickness (mm) of ASD16 46/1 , ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)



4.17d. Study of dry root biomass (g) of ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)

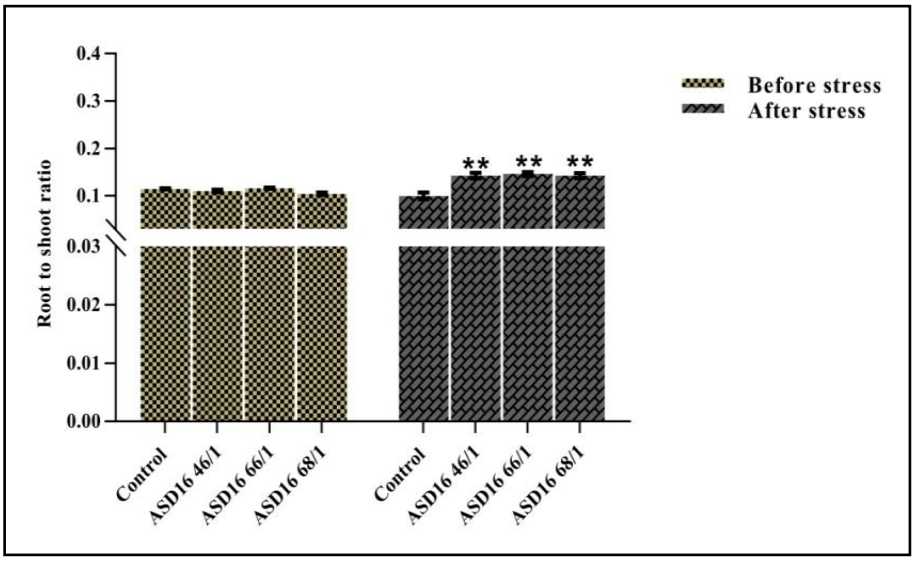


4.17e. Study of dry shoot biomass (g) of ASD16 46/1 , ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05=NS)

significantly increased under drought stress as compared to non-transformed control rice plants (Fig. 4.16). Similarly, Sarkar *et al*., 2016 observed higher root length, root numbers, root dry biomass, root dry biomass and root to shoot ratio in the transgenic peanut (*Arachis hypogaea* L.) lines under drought stress due to overexpression of *AtDREB1A* transcription factor as compared to their non-transformed counterparts. Zhang *et al*., (2017) reported that the transgenic *Nicotiana benthamiana*) plants constitutively expressing *TaPUB1* under the *CaMV35S* promoter as compared to wild type plants showed higher root length under drought stress conditions. Moreover, Rahman *et al*., (2016) reported that the transgenic *indica* rice ASD16 lines overexpressing *EcNAC67* gene showed higher root to shoot biomass under drought and salinity stress conditions. Zheng *et al*. (2017) also reported that the transgenic alfalfa plants expressing Arabidopsis Enhanced Drought Tolerance 1 (*AtEDT1*) gene exhibited larger root systems with larger root lengths, root weight, and root diameters than wild type plants under drought stress by conferring drought tolerance by maximizing water uptake via production of a deeper and more substantial root system. Similarly, transgenic rice, pepper, cotton and poplar plants overexpressing *AtEDT1* showed higher root morphology under drought and salinity stress as reported by (Yu *et al*., 2013, 2016; Zhu *et al*., 2015).

4.5.15. Effect of drought stress on RUBISCO large subunit

Rubisco is the most crucial enzyme in the CO_2 assimilation pathway. Parry *et al.*, (2002) reported that the photosynthetic rate of higher plants is primarily depended on the Rubisco enzyme activity. Several reports suggested that the RUBISCO activity in many crop plants is severely hampered during water stress condition (Bota *et al*., 2004; Vu *et al*, 1999; Inmaculada *et al.,* 2006; Zhou *et al.,* 2007; Feller *et al.,* 2008; Perdomo *et al.,* 2015, 2016). Galmés *et al.,* (2011) suggested that low chloroplastic CO_2 concentration (C_c) occurring under drought stress could induce de-activation of Rubisco in some Mediterranean species. Rubisco actually control photosynthetic carbon assimilation (Reddy *et al.,* 2004). Drought stress induced reduction in the contents and activities of photosynthetic carbon-reduction cycle enzymes, including Rubisco (Maroco *et al.,* 2002; Parry *et al.,* 2002). Zhang *et al.,* (2013) reported that drought stress decreased the synthesis of Rubisco by rapid decline in the abundance of Rubisco large subunit (RbcL) in cucumber. Similarly, Parry *et al.,* (2002) and Chaitanya *et al.*, (2003) also reported the loss in Rubisco activity in several plant species under drought stress. However, Nawaz and Kang (2019) reported that Arabidopsis transgenic plants



4.17 f. Study of root to shoot ratio of ASD16 46/1 , ASD16 66/1 and ASD16 68/1 transgenic lines and non-transformed control plant before and after drought stress (P value < 0.05 = *, < 0.01 =**, <0.001 =***, >0.05 =NS)

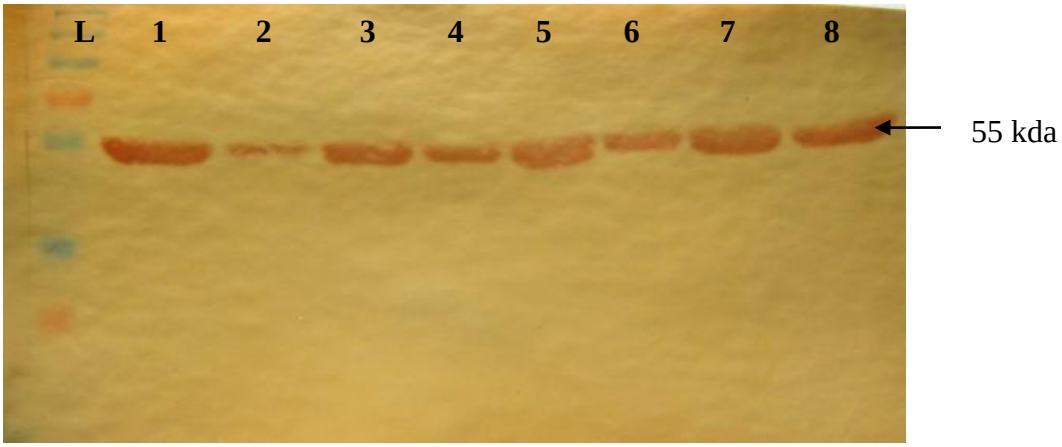


Fig. 4.18: Western blot analysis of RUBISCO large subunit protein in ASD16 46/1, ASD16 66/1 and ASD16 68/1 transgenic lines and nontransformed control plant before and after drought stress. L-Protein ladder (10-250 kda), 1-Control non-stress, 2-Control stress, 3-ASD16 46/1 Non-Stress, 4-ASD16 46/1 Stress, 5- ASD16 66/1 Non-Stress, 6-ASD16 66/1 Stress, 7-ASD16 68/1 Non-Stress, 8-ASD16 68/1 Stress

expressing *OsRH58* transgene showed higher RbcL than that of control non-transformed plants. In the current study, to evaluate if there is any negative effect of *PDH47* transgene in the synthesis and activity of RbcL gene in the transgenic lines before and after drought stress, western blot analysis was carried out. Western blot analysis inferred that *PDH47* gene has no negative effect in the expression of RbcL gene even after exposure to drought stress and it was found that RbcL subunit expression was more in the transgenic lines over expressing *PDH47* transgene as compared to non-transformed rice plants, which might be responsible for improved photosynthetic activity in the transgenic lines (Fig. 4.18). However, Riccardi *et al.,* (2004) reported that the increase quantity of Rubisco protein might be an indirect consequence of reduction in leaf growth rather than a direct response to drought.

Summary and Conclusion…✍

CHAPTER V

SUMMARY AND CONCLUSION

Food security has become one of the major issues, in the current scenario for the continually increasing world population. Besides that, the extreme weather conditions, reduced availability agricultural lands, increased incidence of biotic and abiotic stresses are the limiting factors for crop production. Therefore, it has become imperative to develop sustainable multi-stress tolerant/resistant crop species in order to meet the food demand of the ever growing world population. In order to achieve that, it is vitally important to identify the stress responsive genes which impart tolerance/ resistance in crop plants to a number of abiotic stresses. Among all the abiotic stresses, drought stress generally causes significant loss in crop productivity. In the present study, it has been demonstrated that transgenic rice plants harbouring *PDH47* gene from *Pisum sativum* conferred tolerance to drought stress. The expression of *PDH47* transgene in rice found to increase tolerance of the transgenic rice lines to drought stress with an increased accumulation of osmotic substance proline, maintenance of chlorophyll content, increased relative water content, decreased in ion leakage, decreased in lipid peroxidation (MDA content), lower accumulation of ROS, increased in net photosynthetic rate, stomatal conductance and transpiration rate and maintenance of Rubisco protein even in drought stress condition. Moreover, the transgenic lines showed better root architecture system as compared to that of non-transformed control rice plants. Our findings suggested that overexpression of *PDH47* transgene in cereal crops such as rice would be a suitable strategy to impart tolerance to drought stress conditions.

The salient findings of the research work are summarised below:

1. Genetic transformation of north eastern *indica* rice cultivar Ranjit along with IR64 as control was attempted through *Agrobacterium* mediated method with *PDH47* transgene with an objective to impart drought stress tolerance. *PDH47* transgene was driven by 35S CaMV constitutive promoter and the *hpt* gene was used as a plant selectable marker gene in the transformation protocol.

2. A total of 24 putative transgenic lines were developed for the cultivar IR64. Unfortunately, no transgenic lines of Ranjit could be established in the present study. Out of 24 putative transgenic lines, 19 putative transgenic lines of IR64 were successfully established in the transgenic green house with a regeneration efficiency of 3.59%.

3. Eleven T_0 putative transgenic lines of IR64 showed PCR positive as indicated by presence of *PDH47* and *hptII* transgenes with a transformation efficiency of 2.08% .

4. Three T_3 homozygous transgenic lines gene (ASD16-46/1, ASD16-66/1 and ASD16-68/1) previously developed in the Department of Agricultural Biotechnology were subjected to drought stress treatment for 18 days. The wilting and leaf rolling were found to be less in all the three transgenic rice lines as compared to non-transformed control plants after drought stress.

5. The physiological and biochemical analyses indicated that the expression of *PDH47* gene in the transgenic lines was associated with increased leaf relative water content, water retention capacity, maintenance of chlorophyll, stomatal conductance, net photosynthetic rate, transpiration rate and water use efficiency.

6. These transgenic lines also showed an increased accumulation of the osmolytes like proline, glycine betaine and decreased electrolyte leakage, lipid peroxidation, less accumulation of H_2O_2 during drought stress.

7. The root architecture system of the transgenic rice lines indicated that the expression of *PDH47* transgene was correlated with better root length, root number, root thickness, root dry biomass, shoot dry biomass and root to shoot ratio as compared to that of non-transformed control rice plants after drought stress.

8. Western blotting analysis indicated that RUBISCO large subunit (RbcL) (55KDa) expression was more in the transgenic rice lines as compared to non-transformed control plants after drought stress.

A varying degree of drought stress response was observed in the present study among the three transgenic lines. This study has provided an insight into the regulatory mechanism of action of *PDH47* transgene under drought stress. The detail physiological and biochemical study confirmed that *PDH47* transgene is an important candidate gene which could be introduced into some other cereal crops such as wheat, bajra, millets, *etc*. in order to develop drought stress tolerant/resistant cultivars.

Bibliography …✍

BIBLIOGRAPHY

Abbate, P.E., Dardanellib, J.L., Cantareroc, M.G., Maturanoc, M., Melchiorid, R.J.M. and Sueroa, E.E. (2004). Climatic and water availability effects on water-use efficiency in wheat. *Crop Sci*. **44:** 474–483.

Abdula, S.E., Lee, H.J., Kim, J., Nino, M.C., Jung, Y. J., Cho, Y.C., Nou, I., Kang, K.K. and Cho, Y.G. (2016). BrUGE1 transgenic rice showed improved growth performance with enhanced drought tolerance. *Breeding Science*. **66:** 226 – 233.

Agarwal, M., Sahi, C., Katiyar-Agarwal, S., Agarwal, S., Young, T., Gallie, D.R., Sharma, V., Ganesan, K. and Grover, A. (2002). Molecular characterization of rice hsp101: complementation of yeast hsp104 mutation by disaggregation of protein granules and differential expression in *indica* and *japonica* rice types. *Plant Mol. Biol.* 51: 541–551.

Akram, H. M., Ali, A., Sattar, A., Rehman, H.S.U. and Bibi, A. (2013). Impact of water deficit stress on various physiological and agronomic traits of three basmati rice (*Oryza sativa* L.) cultivars. *J Anim Plant Sci*. **23(5):** 1415–1423.

Aldemita, P.R. and Hodges, T.K. (1996). *Agrobacterium tumefaciens*-mediated transformation of *japonica* and *indica* rice varieties. *Planta*. **199:** 612–617.

Alia, H., Sakamoto, H. and Murata, N. (1998). Enhancment of the tolerance of Arabidopsis to high temperatures by genetic engineering of the synthesis of glycine betaine. *Plant J.* **16 :** 155 -161.

Allen, D.J. and Ort, D.R. (2001). Impact of chilling temperatures on photosynthesis in warm climate plants. *Trends Plant Sci.* **6:** 36-42.

Alt-Moerbe, J., Neddermann, P., Von Lintig, J., Weiler, E.W. and Schroder, J. (1988). Temperature-sensitive step in Ti plasmid vir-region induction and correlation with cytokinin secretion by Agrobacteria. *Mol. Gen. Genet*. **213:**1-8.

Amin, M., Elias, S.M., Hossain, A., Ferdousi, A., Rahman, M. S., Tuteja, N. and Seraj, Z. (2012). Overexpression of a DEAD box helicase, PDH45, confers both seedling and reproductive stage salinity tolerance to rice (*Oryza sativa* L.). *Mol Breeding*. 30(1): 345-54.

Anbazhagan, K., Bhatnagar-Mathur, P., Vadez, V., Dumbala, S. R., Kavi Kishor, P. B. and Sharma, K. K. (2015). *DREB1A* overexpression in transgenic chickpea alters key traits influencing plant water budget across water regimes. *Plant Cell Rep.* **34:** 199–210.

Anjum, F., Yaseen, M., Rasul, E., Wahid, A. and Anjum, S. (2003). Water stress in barley (*Hordeum vulgare* L.). I. Effect on chemical composition and chlorophyll contents. *Pakistan J. Agr. Sci*. **40:** 45–49.

Anjum, S. A., Wang, L. C., Farooq, M., Hussain, M., Xue, L. L. and Zou, C. M. (2011b). Brassinolide application improves the drought tolerance in maize through modulation of enzymatic antioxidants and leaf gas exchange. *J. Agron. Crop Sci.* **197:** 177–185.

Apel, K. and Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol*. **55:** 373–99.

Araus, J. L., Slafer, G. A., Reynolds, M. P. and Royo, C. (2002). Plant breeding and drought in C3 cereals: What should we breed for? *Ann Bot*. **89(7):** 925–940.

Arnon, D. (1949). Copper enzymes isolated chloroplasts, polyphenoloxidase in *Beta vulgaris*. *Plant Physiology*. **24:** 1-15.

Asch, F., Dingkuhnb, M., Sow, A. and Audebert, A. (2005). Drought-induced changes in rooting patterns and assimilate partitioning between root and shoot in upland rice. *Field Crop. Res*. **93:** 223–236.

Ashraf, M. and Foolad, M.R. (2007). Roles of Glycine Betaine and Proline in Improving Plant Abiotic Stress Resistance. *Environmental and Experimental Botany*. **59:** 206-216.

Ashraf, M. and Sharif, R. (1998). Assessment of inter-cultivar/line variation of drought resistance in a potential oil-seed crop, Ethiopian mustard (*Brassica carinata*). *J Plant Nutr*.**43:** 251–265.

Ashraf, M.Y., Naqvi, M.H. and Khan, A.H. (1996).Evaluation of frou screening techniques for drought tolerance in wheat (*Triticum aestivum*). *Acta Agronomica Hungarica*. **44:** 213-220.

Aubourg, S., Kreis, M. and Lecharny, A. (1999). The DEAD box RNA helicase family in *Arabidopsis thaliana. Nucleic Acids Res*. **27:** 628–636.

Augustine, S.M., Narayan, J.A., Syamaladevi, D.P., Appunu, C., Chakravarthi, M., Ravichandran, V., Tuteja, N. and Subramonian, N. (2015). Introduction of Pea DNA Helicase 45 into sugarcane (*Saccharum* spp. Hybrid) enhances cell membrane thermostability and upregulation of stress-responsive genes leads to abiotic stress tolerance. *Mol. Biotechnol*. DOI 10.1007/s12033-015-9841-x.

Azhiri-Sigari, T.A., Yamauchi, A., Kamoshita, A. and Wade, L.J. (2000). Genotypic variation in response of rainfed lowland rice to drought would help to elucidate how rooting depth and deep root and rewatering II. Root growth. *Plant Prod Sci*. **3:** 180–188.

Babita, M., Maheswari, M., Rao, L.M., Shanker, A.K. and Gangadhar Rao, D. (2010). Osmotic adjustment, drought tolerance and yield in castor (*Ricinus communis* L.) hybrids. *Environmental and Experimental Botany*.**69:** 243–249.

Babitha, K.C., Vemanna, R.S., Nataraja, K.N. and Udayakumar, M. (2015). Overexpression of EcbHLH57 Transcription factor from *Eleusine coracana* L. in Tobacco confers tolerance to salt, oxidative and drought stress. *PLos ONE*. **10(9):** e0137098.

Babu, K., Rosaiah, G., Naidu, T.C.M. and Maha Lakshmi , B.K. (2009). Screening of blackgram (Vigna mungo) genotypes for drought tolerance under rain fed conditions. *ANU J. Nat. Sci*. **1(1):** 19–25.

Babu, R.C., Nguyen, B.D., Chamarerk, V.P., Shanmugasundaram, P., Chezhian, P., Jeyaprakash, S.K., Ganesh, A., Palchamy, S., Sadasivam, S., Sarkarung, S., Wade, L.J. and Nguyen, H.T. (2003). Genetic analysis of drought resistance in rice by molecular markers. *Crop Sci*. **43:** 1457–1469.

Bajaj, S. and Mohanty, A. (2005). Recent advances in rice biotechnology-towards genetically superior transgenic rice. *Plant Biotechnology Journal*. 3: 275-307.

Bajji, M., Kinet, J. and Lutts, S. (2002). The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. *Plant Growth Regul*. **36:** 61–70.

Banavath, J.N., Chakradhar, T., Pandit, V., Konduru, S., Guduru, K.K., Akila, C.S., Podha, S. and Puli, C.O.R. (2018). Stress Inducible Overexpression of *AtHDG11* Leads to Improved Drought and Salt Stress Tolerance in Peanut (*Arachis hypogaea* L.). *Front Chem*. 6: 34.

Bao, Y., Aggarwal, P., Robbins, N. E., Sturrock, C. J., Thompson, M. C., Tan, H. Q., Tham, C., Duan, L., Rodriguez, P. L., Vernoux, T., Mooney, S.J., Bennett, M. J. and Dinneny, J. R. (2014). Plant roots use a patterning mechanism to position lateral root branches toward available water. *Proc. Natl. Acad. Sci. U.S.A.* **111:** 9319–9324.

Basu, A., Ray, S., Sarkar, S., Chaudhury, T.R. and Chandu, S. (2014). *Agrobacterium* mediated genetic transformation of popular *indica* rice Ratna (IET1411). *African Journal of Biotechnology.* **13(31):** 3187-3197.

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

Begg, J.E. (1980). Adaptation of plants to water and high temperature stress. In: Turner NC, Kramer PJ (eds) Adaptations of plants to water and high temperature stress. Wiley, New York, pp 33- 42.

Bibi, N., Fan, K., Yuan, S., Ni, M., Ahmed, I. M., Malik, W. and Wang, X. (2013). An efficient and highly reproducible approach for the selection of upland transgenic cotton produced by pollen tube pathway method. *Australian Journal of Crop Sciences.* **7(11):** 1714-1722.

Biswas, A. K. and Choudhuri, M. A. (1984). Effect of water stress at different developmental stages of field-grown rice. *Biol Plant*. **26(4):** 263–266.

Björkman, O. and Demming-Adams, B. (1994). Regulation of photosynthetic light energy capture, conversion, and dissipation in leaves of higher plants. In: Schulze ED, Caldwell MM (eds) Ecophysiology of photosynthesis. Springer, Berlin, pp 17-47.

Blum, A. (1985). Breeding for crop varieties for stress environments. *Crit Rev Plant Sci*. **2:** 199–238.

Blum, A. and Ebercon, A. (1981). Cell membrane stability as a measure of drought and heat tolerance in wheat. *Crop Science*. **21:** 43–47.

Bohnert, H.J. and Jensen, R.G. (1996). Metabolic engineering for increased salt tolerance –the next step. *Aust J plant Physiol.* **23:** 661-667.

Bohnert, H.J., Nelson, D.E. and Jensen, R.G. (1995). Adaptations to environmental stresses. *Plant Cell.* **7:** 1099 -1111.

Bota, J., Flexas, J. and Medrano, H. (2004). Is photosynthesis limited by decreased Rubisco activity and RuBP content under progressive water stress? *New Phytol.* **162:** 671–681.

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

Bray, E.A. (2002). Abscisic acid regulation of gene expression during water deficit stress in the era of the Arabidopsis genome. *Plant Cell Environment.* **25:** 153-161.

Bunnag, S. and Pongthai, P. (2013). Selection of rice (*Oryza sativa* L.) cultivars tolerant to drought stress at the vegetative stage under field conditions. *Am. J. Plant Sci*. **4:** 1701-1708.

Cabuslay, G.S., Ito, O. and Alejar, A.A. (2002). Physiological evaluation of responses of rice (*Oryza sativa* L.) to water deficit. *Plant Science*. **163:** 815-827.

Campo, S., Baldrich, P., Messeguer, J., Lalanne, E., Coca, M. and Segundo, B.S. (2014). Over expression of a Calcium-Dependent Protein Kinase confers salt and drought tolerance in rice by preventing membrane lipid peroxidation. *Plant Physiol*. **165(2) :** 688 – 704.

Centritto, M., Lauteri, M., Monteverdi, M.C. and Serraj, R. (2009). Leaf gas exchange, carbon isotope discrimination, and grain yield in contrasting rice genotypes subjected to water deficits during the reproductive stage. *J Exp Bot*. **60:** 2325–2339.

Chaitanya, K.V., Sundar, D., Jutur, P.P. and Ramachandra Reddy, A. (2003). Water stress effects on photosynthesis in different mulberry cultivars. *Plant Growth Regul*. **40:** 75–80.

Chamot, D., Magee, W.C., Yu, E. and Owttrim, G.W. (1999).A cold shock-induced cyanobacterial RNA helicase. *J. Bacteriol.* **181:** 1728-1732

Chan, M.T., Chang, H.H., Ho, S.L., Tong, W.F. and Yu, S.M. (1993).*Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric α-amylase promoter/β-glucuronidase gene. *Plant Molecular Biology.* 22(3): 491–506.

Chan, M.T., Lee, T.M. and Chang, H.H. (1992). Transformation of *Indica* Rice (*Oryza sativa* L.) Mediated by *Agrobacterium tumefaciens*. *Plant Cell Physiol.* **33(5):** 577-583.

Cha-um, S., Yooyongwech, S. and Supaibulwatana, K. (2010). Water deficit stress in the reproductive stage of four indica rice (*Oryza sativa* L.) genotypes. *Pak. J. Bot*. **42(5):** 3387–3398.

Chen, H., Chen, W., Zhou, J., He, H., Chen, L., Chen, H. and Deng, X. (2012). Basic leucine zipper transcription factor *OsbZIP16* positively regulates drought resistance in rice. *Plant Science.* **193-194:** 8-17.

Chen, J., Qi, T., Hu, Z., Fan, X., Zhu, L., Iqbal, M.F. and Yin, X. (2019). *OsNAR2.1* Positively Regulates Drought Tolerance and Grain Yield Under Drought Stress Conditions in Rice. *Front Plant Sci.* 10: 197.

Chen, L., Marmey, P., Taylor, N.J., Brizard, J.P., Espinoza, C., D'cruz, P., Huet, H., Zhang, S., Kochko, A., Beachy, R.N. and Fauquet, C.M. (1998). Expression and inheritance of multiple transgenes in rice plants. *Nat. Biotechnol.* **16:** 1060-1064.

Chen, M., Wang, Q.Y., Cheng, X.G., Xu, Z.S., Li, L.C., Ye, X.G., Xia, L.Q. and Ma,Y.Z. (2007). *GmDREB2*, a soybean DRE-binding transcription factor, conferred drought and high-salt tolerance in transgenic plants. *Biochemical and Biophysical Research Communications*. **353:** 299–305.

Chen, T. H. and Murata, N. (2002). Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Curr. Opin. Plant Biol*. 5: 250-257.

Chen, T. H. H. and Murata, N. (2008). Glycinebetaine : an effective protectant against abiotic stress in plants. *Trends in Plant Science*. 13: 499-505.

Chinnusamy, V., Jagendorf, A. and Zhu, J.K. (2005). Understanding and improving salt tolerance in plants. *Crop Sci*. **45:** 437–448.

Chinnusamy, V., Schumaker, K. and Zhu, J.K. (2003).Molecular genetics perspectives on cross-talk and specificity in abiotic stress signalling in plants. *Journal of Experimental Botany.* **55**(395): 225-236.

Chiou, T.J. and Bush, D.R. (1998). Sucrose is a signal molecule in assimilate partitioning. *P Natl Acad Sci USA*. **95:** 4784–4788.

Coffman, W.R. and Juliano, B.O. (1987). Rice Nutritional quality of cereal grains, genetic and agronomic improvement. In: *Agronomy Monograph 28*. ASA-CSSA-SSSA, Madison, WI, and USA. p101-31.

Condon, A.G., Richards, R.A., Rebetzke, G.J. and Farquhar, G.D. (2002). Improving intrinsic water-use efficiency and crop yield. *Crop Sci*. **42:** 122-131.

Cordin, O., Tanner, N.K., Doere, M., Linder, P. and Banroques, J. (2004). The newly discovered Q motif of DEAD-box RNA helicases regulates RNA-binding and helicase activity. *EMBO J*. 23: 2478–2487.

Cornic, G. (2000). Drought stress inhibits photosynthesis by decreasing stomatal aperture – not by affecting ATP synthesis. *Trends in Plant Science*. 5: 187-188.

Cornic, G. and Massacci, A. (1996). Leaf photosynthesis under drought stress,in: Baker N.R., (Ed.), Photosynthesis and the Environment, Kluwer Academic Publishers, The Netherlands.

Cruz de Carvalho, M.H. (2008). Drought stress and reactive oxygen species. *Plant Signaling & Behavior*. **3(3):** 156-165.

Cui, K.H., Huang, J.L., Xing, Y.Z., Yu, S.B., Xu, C.G. and Peng, S.B. (2008). Mapping QYLs for seedling characteristics under different water supply conditions in rice (*Oryza sativa*). *Physiol Plant*. **132:** 53–68.

Dansana, P.K., Kothari, K.S., Vij, S. and Tyag, A.K. (2014). *OsiSAP1* overexpression improves water-deficit stress tolerance in transgenic rice by affecting expression of endogenous stress-related genes. *Plant Cell Rep*. **33:** 1425–1440.

Datta, K., Baisakh, N., Ganguly, M., Krishnan, S., Shinozaki, K.Y. and Datta, S. K. (2012). Over expression of Arabidopsis and Rice stress genes inducible transcription factor confers drought and salinity tolerance to rice. *Plant Biotechnology Journal.* **10:** 579-586.

Datta, K., Koukolikova-Nicola, Z., Baisakh, N., Oliva, N. and Datta, S.K.(2000). Agrobacterium mediated engineering for sheath blight resistance of indica rice cultivars from different ecosystems. *Theor. Appl. Genet.* **100:** 832– 839.

Datta, S. K., Peterhans, A., Datta, K. and Potrykus, I. (1990). Genetically engineered fertile Indica rice recovered from protoplasts. *Nat. Biotechnol.* **8:** 736–740.

de la Cruz, J., Kressler, D. and Linder, P. (1999). Unwinding RNA in Saccharomyces cerevisiae: DEAD-box proteins and related families. *Trends Biochem. Sci*. **24:** 192-198.

Del Rio, D., Stewart, A. J. and Pellegrini, N. (2005). A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr. Metab. Cardiovasc. Dis*. **15:** 316–328.

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

Delhaize, E., Ma, J.F. and Ryan, P.R. (2012). Transcriptional regulation of aluminium tolerance genes. *Trends Plant Sci.* **17:** 341–348.

Den Herder, G., Van Isterdael, G., Beeckman, T. and De Smet, I. (2010). The roots of a new green revolution. *Trends Plant Sci.* **15:** 600–607.

Dhanda, S., Sethi, G. and Behl, R. (2004). Indices of drought tolerance in wheat genotypes at early stages of plant growth. *Journal of Agronomy and Crop Science*. **190:** 6–12.

Dietz, K.J., Sauter, A., Wichert, K., Messdaghi, D. and Hartung, W. (2000). Extracellular beta glucosidase activity in barley involved in the hydrolysis of ABA glucose conjugate in leaves. *Journal of Experimental Botany*. **51:** 937-944.

Ding, L., Li, Y. R., Li, Y., Shen, Q. R. and Guo, S. W. (2014). Effects of drought stress on photosynthesis and water status of rice leaves. *Chin J Rice Sci*. **28 (1):** 65–70.

Doyle, J.J. and Doyle, J.L. (1990). Isolation of plant DNA from fresh tissue. *Focus*. **12:** 13–15.

El-Esawi, M.A., Alaraidh, I.A., Alsahli, A.A., Alamri, S.A., Ali, H.M. and Alayafi, A.A. (2018). Bacillus firmus (SW5) augments salt tolerance in soybean (*Glycine max* L.) by modulating root system architecture, antioxidant defense systems and stress-responsive genes expression. *Plant Physiol. Biochem*. **132:** 375–384.

El-Esawi, M.A. and Alayafi, A.A. (2019). Overexpression of Rice Rab7 Gene Improves Drought and Heat Tolerance and Increases Grain Yield in Rice (*Oryza sativa* L.). *Genes*. **10:** 56.

Enriquez-Obregon, G.A., Prieto-Samsonov, D.L., de la Riva,G.A.,Perez, M.I., Selman-Housein, G. and Vazquez-Padron,R. I. (1999). *Agrobacterium*-mediated Japonica rice transformation: a procedure assisted by an antinecrotic treatment. *Plant Cell, Tissue and Organ Culture*. **59 (3):** 159–168.

Escandon, A. and Hahne, G. (1991). Genotypes and composition of culture medium are factors important in the selection for transformed sunflower (*Helianthus annuus*) callus. *Physiol. Plant*. **81:** 367–376.

Estrada-Campuzano, G., Miralles, D.J. and Slafer, G.A. (2008). Genotypic variability and response to water stress of pre- and post-anthesis phases in triticale. *Eur. J. Agron.* **28:** 171-177.

Evenson, R. E., Herdt, R.W. and Hossain, M. (1996). Rice Research in Asia: Progress and Priorities, International Rice Research Institute –CAB International, Wallingford, UK **418p**.

Fahramand, M., Mahmoody, M., Keykha, A., Noori, M. and Rigi, K. (2014).Influence of abiotic stress on proline, photosynthetic enzymes and growth. *Int. Res. J. Appl. Basic Sci*. **8(3):** 257–265.

Faize, M., Burgos, L., Faize, L., Piqueras, A., Nicolas, E., Barba-Espin, G., Clemente-Moreno, M. J., Alcobendas, R., Artlip, T. and Hernandez, J. A. (2011). Involvement of cytosolic ascorbate peroxidase and Cu/Zn superoxide dismutase for improved tolerance against drought stress. *J. Exp. Bot*. **62(8):** 2599–2613.

Farooq, M., Basra, S.M.A., Wahid, A., Cheema, Z.A., Cheema, M.A. and Khaliq, A. (2008). Physiological role of exogenously applied glycinebetaine in improving drought tolerance of fine grain aromatic rice (*Oryza sativa* L.). *J. Agron. Crop Sci.* **194:** 325–333.

Farooq, M., Kobayashi, N., Ito, O., Wahid, A. and Serraj, R. (2010). Broader leaves result in better performance of *indica* rice under drought stress. *J. Plant Physiol*. **167(13):** 1066–1075.

Farooq, M., Wahid, A., Kobayashi, N., Fujita, D. and Basra, S.M.A. (2009). Plant drought stress: effects, mechanisms and management. *Agron. Sustain. Dev.* **29:** 185-212.

Farooq, S. and Azam, F. (2006). The use of cell membrane stability (CMS) technique to screen for salt tolerant wheat varieties. *Journal of Plant Physiology*. **163:** 629–637.

Farrar, J. (1996). Regulation of root weight ratio is mediated by sucrose: Opinion. *Plant Soil*. **185:** 13–19.

Feller, U., Anders, I. and Mae, T. (2008). Rubiscolytics: fate of Rubisco after its enzymatic function in a cell is terminated. *J. Exp. Bot*. **59:** 1615–1624.

Fenta, B.A., Beebe, S.E., Kunert, K.J., Burridge, J.D., Barlow, K.M, Lynch, J.P. and Foyer, C.H. (2014). Field phenotyping of soybean roots for drought stress tolerance. *Agronomy*. **4:** 418–435.

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

Fook, C.W.K., Song, L.K., Yun, W.M. and Mahmood, M. (2015).Efficient regeneration and *Agrobacterium* –mediated transformation protocol for recalcitrant *indica* rice(*Oryza sativa* L.). *Emir. J. Food Agric*. **27(11):** 837-848.

Forde, B.G. (2009). The role of developmental instability in the shaping of a root system. *J. Exp. Bot.* **60:** 3989–4002.

Galmes, J., Ribas-Carbo, M., Medrano, H. and Flexas, J. (2011). Rubisco activity in Mediterranean species is regulated by the chloroplastic CO_2 concentration under water stress. *J. Exp. Bot.* **62 :** 653–665.

Gandhi, R. and Khurana, P. (1999). *Stress-mediated regeneration from mature embryo-derived calli and gene transfer through Agrobacterium in rice (Oryza sativa). Indian Journal of Experimental Biology*. **37 (4):** 332-339.

Gangadhar Rao, D. and Hebbar, K.B. (1996). Lack of relationship between leaf water potential and stomatal conductance in cowpea, maize and sorghum under progressive water stress. *Journal of Plant Physiology and Biochemistry*. **23** (in press).

Gao, W., Bai, S., Li, Q., Gao, C., Liu, G. and Feili, T. (2013). Overexpression of *TaLEA* Gene from *Tamarix androssowii* improves salt and drought tolerance in transgenic poplar (*Populus simonii* × *P. nigra*). *PLoS ONE* **8:** e67462.

Garg, B., Gill, S.S., Biswas, D.K., Sahoo, R.K., Kunchge, N.S., Tuteja, R. and Tuteja, N. (2017). Simultaneous expression of PDH45 with EPSPS gene improves salinity and herbicide tolerance in transgenic tobacco plants. *Frontiers in Plant Science*. **8:** 364.

Gaspar, T., Franck, T., Bisbis, B., Kevers, C., Jouve, L., Hausman, J.F. and Dommes, J. (2002). Concepts in plant stress physiology. Application to plant tissue cultures. *Plant Growth Regul.* **37:** 263-285.

Geda, C.K., Repalli, S.K., Dash, G.K., Swain, P. and Rao, G.J.N. (2019). Enhancement of Drought Tolerance in Rice through Introgression of Arabidopsis *DREB1A* through Transgenic Approach. *J Rice Res*.**7:** 2.

Geigenberger, P., Reimholz, R., Geiger, M., Merlo, L., Canale, V. and **Stitt, M.** (1997). Regulation of sucrose and starch metabolism in potato tubers in response to short-term water deficit. *Planta*. **201:** 502–518.

Ghose, B., Sarker, S., Kpoghomou, M.A., Gao, H., Jun, L., Yin, D. and Ghosh, S. (2013). Self-sufficiency in rice and food security: a South Asian perspective. *Agriculture & Food Security*. **2:** 10.

Gilmour, S.J., Sebolt, A.M., Salazar, M.P., Everard, J.D. and Thomashow, M.F. (2000). Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol*. **124:** 1854–1865.

Gong, Z., Dong, C.H., Lee, H., Zhu, J., Xiong, L., Gong, D., Stevenson, B. and Zhu, J. K. (2005). A DEAD box RNA helicase is essential for mRNA export and important for development and stress responses in Arabidopsis. *Plant Cell*. 17: 256–267.

Gong, Z., Lee, H., Xiong, L., Jagendorf, A., Stevenson, B. and Zhu, J.K. (2002). RNA helicase-like protein as an early regulator of transcription factors for plant chilling and freezing tolerance. Proc. *Natl Acad. Sci,* USA. **99:** 11507 -11512.

Gowda, V. R. P., Henry, A., Yamauchi, A., Shashidhar, H. E. and Serraj, R. (2011). Root biology and genetic improvement for drought avoidance in rice. *Field Crops Res*. **122 (1):**1–13.

Grieve, C.M. and Grattan, S.R. (1983). Rapid Assay for Determination of Water Soluble Quaternary Ammonium Compounds. *Plant and Soil*. **70:** 303-307.

Gui, H., Li, X., Liu, Y. and Li, X. (2016). Evaluation of Factors Impacting Agrobacterium-Mediated Indica Rice Transformation of IR58025B - a Public Maintainer Line. *J Rice Res*. 4: 163.

Guo, L., Wang, Z.Y., Lin, H., Cui, W.E., Chen, J., Liu, M.H., Chen, Z.L., Qu, L.J. and Gu, H.Y. (2006). Expression and functional analysis of the rice plasma-membrane intrinsic protein gene family. *Cell Res*. **16:** 277–286.

Guo, P., Wei, H.X., Zhang, W.J., Yang, B.L. and Bao, Y.J. (2016). The dehydration-induced ERECTA gene, MsSIK1, from alfalfa improved water use efficiency in transgenic Arabidopsis. *Acta Physiol Plant*. **38(2):** 1–12.

Guruprasad, M., Raja, D.S., Jaffar, S.K., Shanthisri, K.V. and Naik, M.S. (2012). An Efficient and Regeneration Protocol for Agrobacterium mediated transformation of *indica* rice. *International journal of Pharmacy and Technology*. **4:** 4280-4286.

Haling, R.E., Brown, L.K., Bengough, A.G., Young, I.M., Hallett, P.D., White, P.J. and George, T.S. (2013). Root hairs improve root penetration, root-soil contact, and phosphorus acquisition in soils of different strength. *J. Exp. Bot*. **64:** 3711–3721.

Hammond, J.P. and White, P.J. (2011). Sugar signaling in root response to low phosphorus availability. *Plant Physiol*. **156:** 1033–1040.

Harris, D., Tripathi, R.S. and Joshi, A. (2002). On-farm seed priming to improve crop establishment and yield in dry direct-seeded rice, in: Pandey S., Mortimer M., Wade L., Tuong T.P., Lopes K., Hardy B. (Eds.),Direct seeding: Research Strategies and Opportunities, International Research Institute, Manila, Philippines, pp 231-240.

Hayat, S., Hayat, Q., Alyemeni, M.N., Wani, A.S., Pichtel, J. and Ahmad, A. (2012). Role of proline under changing environments: a review. *Plant Signal Behav*. **7:** 1456–1466.

Hetherington, A.M. and Woodward, F.I. (2003). The role of stomata in sensing and driving environmental change. *Nature*. **424:** 901–908.

Hiei, Y. and Komari, T. (2008). *Agrobacterium*-mediated transformation of rice using immature embryos or calli induced from mature seed. *Nat. Protoc.* **3:** 824-834.

Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. *The Plant Journal*. **62:** 271-282.

Hossain, M. R., Hassan, L., Patwary, A. K. and Ferdous, M.J. (2009). Optimization of *Agrobacterium tumefaciens* mediated genetic transformation protocol for aromatic rice. *Journal of Bangladesh Agricultural University*. **7:** 235-240.

Hotta, Y., Tanaka, T., Takaoka, H., Takeuchi, Y. and Konnai, M. (1997). New physiological effects of 5-aminolevulinic acid in plants: the increase of photosynthesis, chlorophyll content, and plant growth. *Biosci. Biotechnol. Biochem*. **61(12):** 2025–2028.

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

Htwe, N.N., ChaiLing, H., Zaman, F.Q. **and** Maziah, M. (2014). Plant genetic transformation efficiency of selected Malaysian Rice based on selectable marker gene (hptII). *Pakistan Journal of Biological Sciences*. **17(4):** 472-481.

Huang, C.Z., Li, K.A. and Tong, S.Y. (1997). Determination of nanograms of nucleic acids by their enhancement effect on the resonance light scattering of the cobalt (II)/4-[(5- chloro-2-pyridyl) azo]-1, 3- diaminobenzene complex. *Analytical chemistry*. **69(3):** 514-520.

Huang, G. T., Ma, S. L., Bai, L. P., Zhang, L., Ma, H., Jia, P., Liu, J., Zhong, M. and Guo, Z. F. (2012). Signal transduction during cold, salt, and drought stresses in plants. *Mol. Biol. Rep.* **39:** 969–987.

Huang, J., Sun, S.J., Xu, D.Q., Yang, X., Bao, Y.M., Wang, Z.F., Tang. H.J. and Zhang, H. (2009). Increased tolerance of rice to cold, drought and oxidative stresses mediated by the over expression of a gene that encodes the zinc finger protein ZFP245. *Biochemical and Biophysical Research Communications*. **389:** 556–561.

Hussain, S.S. (2006). Molecular breeding for abiotic stress tolerance: drought perspective. *Proc Pakistan Acad Sci*. **43(3):** 189–210.

Inmaculada, J., Navarro, R.M., Lenz, C., Ariza, D. and Jorrin, J. (2006). Variation in the holm oak leaf proteome at different plant developmental stages, between provenances and in response to drought stress. *Proteomics*. **6:** 207-214.

International Rice Research Institute (2013). Rice knowledge bank. International Rice Research Institute, Los Baños, Philippines.

Jain, R.K. (1997). Effects of some factors on plant regeneration from *indica* rice cells and protoplasts— A review. *Indian J. Exp. Biol.* **35:** 323-331.

Jaleel, C.A., Gopi, R. and Panneerselvam, R. (2008b). Growth and photosynthetic pigments responses of two varieties of *Catharanthus roseus* to triadimefon treatment. *Comp. Rend. Biol*. **331:** 272–277.

Jaleel, C.A., Gopi, R., Sankar, B., Gomathinayagam, M. and Panneerselvam, R. (2008a). Differential responses in water use efficiency in two varieties of *Catharanthus roseus* under drought stress. *Comp. Rend. Biol*. **331:** 42–47.

Jaleel, C.A., Manivannan, P., Lakshmanan, G.M.A., Gomathinayagam, M. and Panneerselvam, R. (2008c). Alterations in morphological parameters and photosynthetic pigment responses of *Catharanthus roseus* under soil water deficits.*Colloids Surf. B: Biointerfaces.* **61:** 298-303.

Jaleel, C.A., Manivannan, P., Murali, P.V., Gomathinayagam, M. and Panneerselvam, R. (2008d). Antioxidant potential and indole alkaloid profile variations with water deficits along different parts of two varieties of *Catharanthus roseus*. *Colloids Surf. B: Biointerfaces*. **62:** 312–318.

Jaleel, C.A., Sankar, B., Murali, P.V., Gomathinayagam, M., Lakshmanan, G.M.A. and Panneerselvam, R. (2008e).Water deficit stress effects on reactive oxygen metabolism in *Catharanthus roseus;* impacts on ajmalicine accumulation. *Colloids Surf. B: Biointerfaces.* **62:** 105-111.

Jaleel, C.A., Manivannan, P., Sankar, B., Kishorekumar, A., Gopi, R., Somasundaram, R. and Panneerselvam, R. (2007d). Induction of drought stress tolerance by ketoconazole in *Catharanthus roseus* is mediated by enhanced antioxidant potentials and secondary metabolite accumulation. *Colloids Surf. B: Biointerfaces.* **60:** 201-206.

Janna, O.A., Maziah, M. and Parvezz, G.K.A. (2000). Research note: Potential selective agent for orchid transformation. *Asia Pacific J. Mol. Biol. Biotech*. **8:** 85-93.

Ji, K., Wanga, Y., Sun, W., Lou, Q., Mei, H., Shen, S. and Chen, H. (2012). Drought-responsive mechanisms in rice genotypes with contrasting drought tolerance during reproductive stage. *Journal of Plant Physiology*. **169:** 336– 344.

Jiang,Y., Qiu,Y., Yanru Hu, Y. and Yu, D. (2016). Heterologous Expression of AtWRK57 Confers Drought Tolerance in *Oryza sativa*. *Front. Plant Sci.* 7: 145.

Jones, H.G. (1993). Drought Tolerance and Water-use Efficiency. In: Water Deficits Plant Responses from Cell to Community, Smith, J.A.C. and H. Griffiths (Eds.). *Bios Scientific Publishers Ltd.*, Oxford, pp: 193-204.

Jones, H.G. and Jones, M.B. (1989).Introduction: some terminology and common mechanisms. Plant under Stress, Cambridge University Press, Cambridge. pp. 1-10.

Joshi, R., Sahoo, K.K., Singh, A.K., Anwar, K., Pundir, P., Gautam, R.K., Krishnamurthy, S.L., Sopory, S.K., Pareek, A. and Singla-Pareek, S.L. (2020). Enhancing trehalose biosynthesis improves yield potential in marker-free transgenic rice under drought, saline, and sodic conditions. *J. Exp. Bot.* **71(2):** 653–668.

Karaba, A., Dixit, S., Greco, R., Aharoni, A., Trijatmiko, K.R., Martinez, N.M., Krishnan, A., Nataraja, K.N., Udayakumar, M. and Pereira, A. (2007). Improvement of water use efficiency in rice by expression of *HARDY*, an *Arabidopsis* drought and salt tolerance gene. *PNAS*. **104(39) :** 15270 – 15272.

Karthikeyan, A., Pandian, S.K. and Ramesh, M. (2011). *Agrobacterium*-mediated transformation of leaf base derived callus tissues of popular *indica* rice (*Oryza sativa* L. subsp. *indica* cv. ADT 43). *Plant Science*. **181:** 258 – 268.

Kathiresan, A., Lafitte, H.R., Chen, J.X., Mansueto, L., Bruskiewich, R. and Bennett, J. (2006). Gene expression microarrays and their application in drought stress research. *Field Crop Res*. **97:** 102–110.

Kathuria, H., Giri, J., Nataraja, K.N., Murata, N., Udayakumar, M. and Tyagi, A.K. (2009). Glycinebetaine-induced water-stress tolerance in codA-expressing transgenic *indica* rice is associated with up-regulation of several stress responsive genes. *Plant Biotechnology Journal*. **7 :** 512–526.

Kavi Kishor, P.B., Hong, Z., Miao, C.H., Hu, C.A.A and Verma, D.P.S. (1995). Overexpression of A1-Pyrroline-5-Carboxylate Synthetase lncreases Proline Production and Confers Osmotolerance in Transgenic Plants. *Plant Physiol.* 108: 1387-1394.

Kaya, C. Higgs, D., Ince, F., Amador, B.M., Cakir, A. and Sakar, E. (2003). Ameliorative effects of potassium phosphate on salt-stressed pepper and cucumber. *Journal of Plant Nutrition*, **26:** 807-820.

Kaya, M.D., Okcub, G., Ataka, M., Cikihc, Y. and Kolsaricia, O. (2006). Seed treatments to overcome salt and drought stress during germination in sunflower (*Helianthus annuus* L.). *Eur. J. Agron.* **24:** 291-295.

Khanna, H.K. and Raina, S.K. (2002). Elite *Indica* Transgenic Rice Plants Expressing Modified Cry1Ac Endotoxin of *Bacillus thuringiensis* Show Enhanced Resistance to Yellow Stem Borer (*Scirpophaga incertulas*). *Transgenic Research*. 11: 411-423.

Khush, G.S. (1999). Green revolution: preparing for the 21st century, *Genome* **42 :** 646-655.

Kohli, A., Miro, B. and Twyman, R. M. (2010). Transgene integration, expression and stability in plants: strategies for improvements. *Transgenic Crop Plants*. pp 201-237.

Komari, T., Hiei, Y., Saito, Y., Murai, N. and Kumashiro, T. (1996).Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. *The Plant Journal*. 10: 165–174.

Kumar, K.K., Maruthasalam, S., Loganathan, M., Sudhakar, D. and Balasubramanian, P. (2005). An improved *Agrobacterium* mediated transformation protocol for recalcitrant elite *indica* rice cultivars. *Plant Molecular Biology Reporter*. **23:** 67-73.

Kumar, S., Dwivedi, S. K., Singh, S. S., Bhatt, B. P., Mehta, P., Elanchezhian, R., Singh, V. P. and Singh, O. N. (2014). Morphophysiological traits associated with reproductive stage drought tolerance of rice (*Oryza sativa* L.) genotypes under rain-fed condition of eastern Indo-Gangetic Plain. *Ind. J. Plant Physiol*. **19 (2):** 87–93.

Lanceras, J.C., Pantuwan, G., Jongdee, B. and Toojinda, T. (2004). Quantitative trait loci associated with drought tolerance at reproductive stage in rice. *Plant Physiol*. **135:** 384–399.

Latha, G.M., Raman, K.V., Lima, J.M., Pattanayak, D., Singh, A.K., Chinnusamy, V., Bansal, K.C., Sambasiva Rao, K.R.S. and Mohapatra, L. (2019).Genetic engineering of indica rice with AtDREB1A gene for enhanced abiotic stress tolerance. *Plant Cell, Tissue and Organ Culture*. **136:** 173-188.

Lee, B. and Zhu, J.K. (2010). Phenotypic analysis of Arabidopsis mutants: electrolyte leakage after freezing stress. *Cold Spring Harbour Protocols*. pdb.prot4970.

Lemoine, R., Camera, S.L., Atanassova, R., De´dalde´champ, F., Allario, T., Pourtau, N., Bonnemain, J.L., Laloi, M., Coutos-The´venot, P., Maurousset, L., Faucher, M., Girousse, C., Lemonnier, P., Parrilla, J. and Durand, M. (2013). Source-to-sink transport of sugar and regulation by environmental factors. *Front Plant Sci*. **4:** 272.doi: 10. 3389/fpls.2013.00272.

Leopold, A.C., Musgrave, M.E. and Williams, K.M. (1981). Solute leakage resulting from leaf desiccation. *Plant Physiology*. **68:** 1222–1225.

Levitt, J. (1972). Responses of plants to environmental stresses. London: Academic Press.

Li, H., Han, X., Liu, X., Zhou, M., Ren, W., Zhao, B., Ju, C., Liu, Y. and Zhao, J. (2019). Leucine-rich repeat receptor like kinase gene SbER2 -1 from sorghum (*Sorghum bicolour* L.) confers drought tolerance in maize. *BMC Genomics*. **20:** 737.

Lin, Y.J. and Zhang, Q. (2005). Optimizing the tissue culture conditions for high efficiency transformation system using the biolistic method. *Plant Cell Reports*. **12:** 250-255.

Linder, P., Lasko, P.F., Ashburner, M., Leroy, P., Nielsen, P.J., Nishi, K., Schnier, J. and Slonimski, P. P. (1989). Birth of the D-E-A-D box. *Nature*. 337: 121–122.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1998). Two transcription factors, DREB1 and DREB2, with an EREBP / AP2 DNA binding domain separate two cellular signal transduction pathways in drought-and low-temperature-responsive gene expression, respectively, in Arabidopsis. *Plant Cell*. **10:** 1391–1406.

Loresto, G.C. and Chang, T.T. (1981). Decimal scoring systems for drought reaction and recovery ability in rice screening nurseries. *Int. Rice Res. Newsletter*. **6(2) :** 9-10.

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

Lu, Z., Percy, R.G., Qualset, C.O. and Zeiger, E. (1998).Stomatal conductance predicts yield in irrigated Pima cotton and bread wheat grown at high temperatures. *Journal of Experimental Botany*. **49:** 453–460.

Luan, S., Kudla, J., Rodriguez-Concepcion, M., Yalovsky, S. and Gruissem, W. (2002). Calmodulins and calcineurin B-like proteins: calcium sensors for specific signal response coupling in plants. *Plant Cell*. **14:** S389–S400.

Luck, H. (1974). In: Methods in enzymatic analysis. 2nd ed. New York : Bergmeyer Academic Press.p.805.

Luking, A., Stahl, U. and Schmidt, U. (1998). The protein family of RNA helicases. *Critical Review of Biochemical and Molecular Biology*. **33:** 259 - 296.

Lum, M .S., Hanafi, M. M., Rafii, Y. M. and Akmar, A.S. N. (2014). Effect of drought stress on growth, proline and antioxidant enzyme activities of upland rice. *J. Anim. Plant Sci*. **24**(5): 1487–1493.

Lynch, J. (1995). Root architecture and plant productivity. *Plant Physiol.* **109:** 7–13.

Lynch, J.P. (2007). Roots of the second green revolution. *Aust. J. Bot.* **55:** 493–512.

Macovei, A., Vaid, N., Tula, S. and Tuteja, N. (2012). A new DEAD-box helicase ATP-binding protein (OsABP) from rice is responsive to abiotic stress. *Plant Signal. Behav.* **7:**1138–1143.

Madhusudan, K.V., Giridarakumar, S., Ranganayakulu, G.S., Chandraobulreddy, P. and Sudhakar, C. (2002). Effect of water stress on some physiological responses in two groundnut (*Arachis hypogaea* L.) cultivars with contrasting drought tolerance. *J. Plant Biol*. **29:** 199-202.

Maisura, Chozin, M. A., Lubis, I., Junaedinand, A. and Ehara, H. (2014). Some physiological character responses of rice under drought conditions in a paddy system. *J. Int. Soc. Southeast Asian Agric. Sci*. **20 (1):** 104–114.

Manavalan, L.P., Guttikonda, S.K., Nguyen, V.T., Shannon, J.G. and Nguyen, H.T. (2010). Evaluation of diverse soybean germplasm for root growth and architecture. *Plant Soil.* **330:** 503–514.

Manikavelu, A., Nadarajan, N., Ganesh, S.K., Gnanamalar, R.P. and Babu, R.C. (2006). Drought tolerance in rice: morphological and molecular genetic consideration. *Plant Growth Regul.* **50:** 121-138.

Marco, F., Bitrián, M., Carrasco, P., Rajam, M.V., Alcázar, R. and Tiburcio, A.F. (2015). Genetic engineering strategies for abiotic stress tolerance in plants. In: Bahadur B, Rajam MV, Sahijram L, Krishnamurthy KV (eds) Plant biology and biotechnology. Springer, NY, pp 579–609.

Maroco, J.P., Rodrigues, M.L., Lopes, C. and Chaves, M.M. (2002). Limitations to leaf photosynthesis in field-grown grapevine under drought-metabolic and modeling approaches. *Funct. Plant Biol*. **29:** 451-459.

Matson, S. W. (1991). DNA helicases of Escherichia coli. Prog. *Nucleic Acids Res. Mol. Bio.* **40:** 289-326.

Mbinda, W., Ombori, O., Dixelius, C. and Oduor, R. (2018). *Xerophyta viscosa* aldose reductase, *XvAld1*, enhances drought tolerance in transgenic sweetpotato. *Mol. Biotechnol.* **60:** 203–214.

Mellacheruvu, S., Tamirisa, S., Vudem, D.R. and Khareedu, V.R. (2016). Pigeon pea hybrid proline rice protein (CcHyPRP) confers biotic and abiotic stress tolerance in transgenic rice. *Front Plant Sci*. **6:** 1167.

Meloni, D.A., Olivamartinez, M.A. and Cambraia, J. (2003). Photosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. *Environmental and Experimental Botany*. **49:** 69-76.

Menconi, M., Sgherri, C.L.M., Pinzino, C. and Navari-Izzo, F. (1995). Activated oxygen production and detoxification in wheat plants subjected to a water deficit programme. *J. Exp. Bot*. **46:**1123-1130.

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci*. **7:** 405–410.

Mohanty, A., Kathuria, H., Ferjani, A., Sakamoto, A., Mohanty, P., Murata, N. and Tyagi, A.K. (2002). Transgenics of an elite *indica* rice variety Pusa Basmati 1 harbouring the *codA* gene are highly tolerant to salt stress. *Theor Appl Genet*. 106: 51-57.

Moller, I.M., Jensen, P.E. and Hansson, A. (2007). Oxidative modifications to cellular components in plants. *Annu. Rev. Plant Biol.* **58:** 459-481.

Mostajeran, A. and Rahimi –Eichi, V. (2009). Effects of drought stress on growth and yield of rice (*Oryza sativa* L.) cultivars and accumulation of proline and soluble sugars in sheath and blades of their different ages leaves. *Am-Eur. J. Agric. Environ. Sci*. **5 (2):** 264–272.

Mostofa, M.G. and Fujita, M. (2013). Salicylic acid alleviates copper toxicity in rice (*Oryza sativa* L.) seedlings by up-regulating antioxidative and glyoxalase systems. *Ecotoxicology.* 22: 659–681.

Murtaza, G., Rasool, F., Habib, R., Javed, T., Sardar, K., Muhammad Mohsin Ayub, M.M., Ayub, A.M. and Rasool, A. (2016). A review of Morphological, Physiological and Biochemical responses of plants under drought stress conditions. *Imperial Journal of Interdisciplinary Research.* **12(2) :** 2454-1362.

Muzny, D.M. *et al*. (2006). The DNA sequence, annotation and analysis of human chromosome 3. *Nature*. **440(7088):** 1194-1198.

Nakano, Y. and Asada, K. (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant and Cell Phyiology.* **22:** 867-880.

Nandakumar, R., Babu, S., Kalpana, K., Raguchander, T., Balasubramanian, P. and Samiyappan, R. (2007). *Agrobacterium* mediated transformation of *indica* rice with chitinase gene for enhanced sheath blight resistance. *Biologia Plantarum.* **51(1) :**142-148.

Nawaz, G. and Kang, H. (2019). Rice OsRH58, a chloroplast DEAD-box RNA helicase, improves salt or drought stress tolerance in *Arabidopsis* by affecting chloroplast translation. *BMC Plant Biology*. **19:**17.

Nayak, K.P., Basu, D., Das, S., Basu, A., Ghosh, D., Ramakrishna, N. A., Ghosh, M. and Sen, S.K. (1997). Transgenic elite *indica* rice plants expressing *Cry1Ac* delta- endotoxin of *Bacillus thuringiensis* are resistant against yellow stem borer (*Scirpophaga incertulas*). *Proc. Natl. Acad. Sci*. **94:** 2111-2116.

Nayyar, H. and Gupta, D. (2006). Differential sensitivity of C_3 and C_4 plants to water deficit stress: association with oxidative stress and antioxidants. *Environ. Exp. Bot*. **58:** 106-113.

Nelson, D., Repetti, P.P., Adams, T.R., Creelman, R.A., Wu, J., Warner, D.C., Anstrom, D.C., Bensen, R.J., Castiglioni, P.P., Donnarummo, M.G., Hinchey, B.S., Kumimoto,R.W., Maszle, D.R., Canales,R.D., Krolikowski,K.A., Dotson,S.B., Gutterson, N., Ratcliffe, O.J. and Heard, J.E. (2007). Plant nuclear factor Y (NF-Y) B subunits confer drought tolerance and lead to improved corn yields

on water-limited acres. *Proceedings of the National Academy of Sciences*. **104:** 16450–16455.

Newton, A.C., Johnson, S.N. and Gregory, P.J. (2011). Implications of climate change for diseases, crop yields and food security. *Euphytica*. **179:** 3-18.

Ngachan, S. V., Mohanty, A. K. and Pattanayak, A. (2011). Status paper on rice in North east India.ICAR Research Complex for North-East Hill Region. pp 1.

Nidumukkala, S., Tayi, L., Chittela, K. R., Vudem, D.R., Rao, V. and Khareedu, V.R. (2019). DEAD box helicases as promising molecular tools for engineering abiotic stress tolerance in plants. *Critical Reviews in Biotechnology*. **39(3):** 395-407.

Noctor, G. and Foyer, C. H. (1998). Ascorbate and glutathione: Keeping active oxygen under control. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **49**: 249–279.

Nyaboga, E., Tripathi, J.N., Manoharan, R. and Tripathi, L. (2014). *Agrobacterium*-mediated genetic transformation of yam (*Dioscorea rotundata*): an important tool for functional study of genes and crop improvement. *Front Plant Sci.* **5:** 463.

O'Toole, J.C. and Chang, T.T. (1978). Drought and rice improvement in perspective. IRRI research paper series, No.14.

Olhoft, P. and Somers, D. (2001). L-Cysteine increases Agrobacterium-mediated T-DNA delivery into soybean cotyledonary-node cells. *Plant Cell Reports*. 20(8): 706-711.

Osorio, S., Alba, R., Damasceno, C. M. B., Lopez-Casado, G., Lohse, M., Zanor, M. I., Tohge, T., Usadel, B., Rose, J.K.C., Fei, Z., Giovannoni, J.J. and Fernie, A. R. (2011). Systems biology of tomato fruit development: combined transcript, protein and metabolite analysis of tomato transcription factor (nor, rin) and ethylene receptor (Nr) mutants reveals novel regulatory interactions. *Plant Physiol*. 157: 405–425.

Padilla, I. M. and Burgos, L. (2010). Aminoglycoside antibiotics: structure, functions and effects on in vitro plant culture and genetic transformation protocols. *Plant Cell Rep.* **29:** 1203–1213.

Pardo, J.M. (2010). Biotechnology of water and salinity stress tolerance. *Curr Opin Biotechnol*. 21:185–196.

Parida, B.R. and Oinam, B. (2015).Unprecedented drought in North East India compared to Western India. *Current Science.* **109:** 2121-2126.

Parry, M.A.J., Andralojc, P.J., Khan, S., Lea, P. J. and Keys, A. J. (2002). Rubisco activity: effects of drought stress. *Annals of Botany*. **89:** 833–839.

Parry, M.A.J., Flexas, J. and Medrano, H. (2005). Prospects for crop production under drought: research priorities and future directions. *Ann Appl Biol*. **147:** 211–226.

Perdomo, J. A., Conesa, M. A., Medrano, H., Ribas-Carbo, M. and Galmes J. (2015). Effects of long-term individual and combined water and temperature stress on the growth of rice, wheat and maize: relationship with morphological and physiological acclimation. *Physiol. Plant.* **155:** 149–165.

Perdomo, J. A., Carmo-Silva, E., Hermida-Carrera, C., Flexas, J. and Galmes, J. (2016). Acclimation of biochemical and diffusive components of photosynthesis in rice, wheat and maize to heat and water deficit: implications for modeling photosynthesis. *Front. Plant Sci*. **7:**1719.

Pieters, A. J. and Souki, S. E. (2005). Effects of drought during grain filling on PSII activity in rice. *J Plant Physiol*. **162(8):** 903–911.

Pinto, L. M. N., Fiuza, L.M., Ziegler, D., De Oliveira, J.V., Menezes, V.G., Bourrie, I., Meynard, D., Guiderdoni, E., Breitler, J.C., Altosaar, I. and Gantet, P. (2013). *Indica* Rice cultivar IRGA424, transformed with cry genes of *B. Thuringiensis*, provided high resistance against *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *Journal of Economic Entomology*. **106:** 2585 – 2594.

Pirdashti, H., Sarvestani, Z. T. and Bahmanyar, M. A. (2009). Comparison of physiological responses among four contrast rice cultivars under drought stress conditions. *Proc. World Acad. Sci. Engin. Technol*. **49:** 52–53.

Poorter, H., Niklas, K.J., Reich, P.B., Oleksyn, J., Poot, P. and Mommer, L. (2012). Biomass allocation to leaves, stems and roots: meta-analyses of interspecific variation and environmental control. *New Phytol.* **193 (1):** 30-50.

Premachandra, G.S., Saneoka, H. and Ogata, S. (1991). Cell membrane stability and leaf water relations as affected by potassium nutrition of water-stressed maize. *J. Exp. Bot.* **42**: 739–74.

Prince, S.J., Mutava, R.N., Pegoraro, C., Oliveira, A.C.D. and Nguyen, H.T. (2013). Root characters. In Genomics and Breeding for Climate Resilient Crops; Kole, C., Ed.; Springer: Berlin, Germany. pp. 67–131.

Priya, A.M., Krishnan, S.R. and Ramesh, M. (2015). Ploidy stability of *Oryza sativa*. L cv IR64 transformed with the moth bean P5CS gene with significant tolerance against drought and salinity. *Turk J Biol*. **39:** 407-416.

Priyanka, B., Sekhar, K., Reddy, V.D. and Rao, K.V. (2010a). Expression of pigeonpea hybrid-proline-rich protein encoding gene (CcHyPRP) in yeast and Arabidopsis affords multiple abiotic stress tolerance. *Plant Biotechnol J*. **8:** 76-87.

Priyanka, B., Sekhar, K., Sunita, T., Reddy, V.D. and Rao, K.V. (2010b). Characterization of expressed sequence tags (ESTs) of pigeonpea (*Cajanus cajan* L.) and functional validation of selected genes for abiotic stress tolerance in Arabidopsis thaliana. *Mol Genet Genomics.* **283:** 273 -287.

Puertolas, J., Conesa, M.R., Ballester, C. and Dodd, I.C. (2014).Local root abscisic acid (ABA) accumulation depends on the spatial distribution of soil moisture in potato: implications for ABA signalling under heterogeneous soil drying. *Journal of Experimental Botany*. **66:** 2325-34.

Quan, R.D., Shang, M., Zhang, H. and Zhang, J. (2004) Improved chilling tolerance by transformation with betA gene for the enhancement of glycine betaine synthesis in maize. *Plant Sci.* **166:** 141–149.

Rahman, H., Ramanathan, V., Nallathambi, J., Duraialagaraja, S. and Muthurajan, R. (2016). Over-expression of a NAC 67 transcription factor from finger millet (*Eleusine coracana* L.) confers tolerance against salinity and drought stress in rice. *BMC Biotechnol*. 16: 35.

Raineri, D.M., Bottino, P., Gordon, M.P. and Nester, E.W. (1990).Agrobacterium-mediated transformation of rice (*Oryza sativa* L.). *Nature Biotechnology*. 8: 33-38.

Ramanjulu, S. and Sudhakar, C. (1997). Drought tolerance is partly related to amino acid accumulation and ammonia assimilation: A comparative study in two mulberry genotypes differing in drought sensitivity. *Journal of Plant Physiology*. **150:** 345–350.

Rashid, H., Yokoi, S., Toriyama, K. and Hinata, K. (1996). Transgenic plant production mediated by *Agrobacterium* in *indica* rice. *Plant Cell Rep*. **15:** 727-730.

Rathinasabapathi, B., Sigua, C., Ho, J. and Gage, D.A. (2000). Osmoprotectant β-alanine betaine synthesis in the Plumbaginaceae: S-adenosyl-L-methionine dependent N methylation of β-alanine to its betaine via N-methyl and N, N-dimethyl β-alanines. *Physiol Plant*. **109:** 225–231.

Ratnakumar, P., Vadez, V., Nigam, S.N. and Krishnamurthy, L. (2009). Assessment of transpiration efficiency in peanut (*Arachis hypogaea* L.) under drought using a lysimetric system. *Plant Biology.* **11:**124–130.

Ravikumar, G., Manimaran, P., Voleti, S.R., Subrahmanyam, D., Sundaram, R.M., Bansal, K.C., Viraktamath, B.C. and Balachandran, S.M. (2014). Stress inducible expression of AtDREB1A transcription factor greatly improves drought stress tolerance in transgenic *indica* rice. *Transgenic Res*. **23:** 421-439.

Reddy, A. R., Chaitanya, K. V. and Vivekanandan, M. (2004). Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. *J. Plant Physiol.* **161:** 1189–1202.

Reddy, K.J. and Vanaja, M. (2006). Plant responses to drought stress. *Journal of Plant Biology*. **33:** 115-124.

Rejeb, I.B., Pastor, V. and Mauch-Mani, B. (2014). Plant Responses to Simultaneous Biotic and Abiotic Stress: Molecular Mechanisms. *Plants*. **3:** 458-475.

Reynolds, M. and Tuberosa, R. (2008). Translational research impacting on crop productivity in drought-prone environments. *Curr Opin Plant Biol*. **11:** 171–179.

Rhodes, D. and Samaras, Y. (1994). Genetic control of osmoregulation in plants. *In*: Strange S K. Cellular and Molecular Physiology of Cell Volume Regulation. Boca Raton: CRC Press: 347–361.

Riccardi, F., Gazeau, P., Jacquemot, M.P., Vincent, D. and Zivy, M. (2004). Deciphering genetic variation of proteome responses to water deficit in maize leaves. *Plant Physiol Biochem*. **42:** 1003–1011.

Rich, S. M. and Watt, M. (2013). Soil conditions and cereal root system architecture: review and considerations for linking Darwin and Weaver. *J. Exp. Bot.* **64:** 1193–1208.

Robbins, N.E. and Dinneny, J.R. (2015). The divining root: moisture-driven responses of roots at the micro- and macro-scale. *J. Exp. Bot.* 66(8): 2145–2154.

Rocak, S. and Linder, P. (2004). DEAD-box proteins: the driving forces behind RNA metabolism.

Roel, A., Heilman, J.L. and McCauley, G.N. (1999). Water use and plant response in two rice irrigation methods. *Agric. Water Manage*. **39:** 35-46.

Rosellini, D., Capomaccio, S., Ferradini, N., Sardaro, M. L. S., Nicolia, A. and Veronesi, F. (2007). Non-antibiotic, efficient selection for alfalfa genetic engineering. *Plant Cell Rep.* **26:** 1035–1044.

Sahoo, K.K., Tripathi, A.K., Pareek, A., Sopory, S.K. and Singla-Pareek, S.L. (2011). An improved protocol for efficient transformation and regeneration of diverse *indica* rice cultivars. *Plant Methods.* **7(49):** 1-11.

Sahoo, R.K., Gill, S.S. and Tuteja, N. (2012). Pea DNA helicase 45 promotes salinity stress tolerance in IR64 rice with improved yield. *Plant Signal Behav*. 7(8): 1042–1046.

Sakamoto, A. and Murata, N. (2001). The use of bacterial choline oxidase , a glycine betaine – synthesizing enzyme to create stress – resistance transgenic plants. *Plant Physiology*. **125:** 180 -188.

Sakamoto, A. and Murata, N. (2002). The role of glycinebetaine in the protection of plants from stress: clues from transgenic plants. *Plant Cell Environ.* **25:** 163–171.

Sakhanokho, H.F., Ozias-akins, P., May, O.L. and Chee, P. (2004). Induction of somatic embryogenesis and plant regeneration in select Georgia and Pee Dee cotton lines. *Crop Science*. **44:** 2199-2205.

Sambrook, J., Fritschi, E.F. and Maniatis, T. (1989). Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, New York.

Sánchez, F. J., Manzanares, M., de Andres, E. F., Tenorio, J. L. and Ayerbe, L. (1998). Turgor maintenance, osmotic adjustment and soluble sugar and proline accumulation in 49 pea cultivars in response to water stress. *Field Crop Res.* **59:** 225–235.

Sarangi, S., Ghosh, J., Bora, A., Das, S. and Mandal, A. B. (2009). *Agrobacterium* mediated genetic transformation of indica rice varieties involving Am-SOD gene. *Indian Journal of Biotechnology.* **10:** 9-18.

Sarkar, T., Thankappan, R., Kumar, A., Mishra, G.P. and Dobaria, J.R. (2016). Stress Inducible Expression of *AtDREB1A* Transcription Factor in Transgenic Peanut (*Arachis hypogaea* L.) Conferred Tolerance to Soil-Moisture Deficit Stress. *Front. Plant Sci*. **7:** 935.

Schachtman, D.P. and Goodger, J.Q. (2008). Chemical root to shoot signalling under drought. *Trends in Plant Science*. **13:** 281-287.

Selote, D. S. and Khanna-Chopra, R. (2004). Drought-induced spikelet sterility is associated with an inefficient antioxidant defence in rice panicles. *Physiol Plant*. **121(3):** 462–471.

Serraj, R., Kumar, A., McNally, K.L., Slamet-Loedin, I., Bruskiewich, R., Mauleon, R., Cairns, J. and Hijmans, R.J. (2009). Improvement of drought resistance in rice. *Adv. Agron*. **103:** 41-99.

Serraj, R., Hash, C. T., Rizvi, M. H. S., Sharma, A., Yadav, R. S. and Bidinger, F. R. (2005). Recent advances in marker assisted selection for drought tolerance in pearl millet. *Plant Prod. Sci.* **8:** 334–337.

Seth, M. S., Bedada, L. T., Runo, S. M., Ngugi, M. P., Iyer, R., Thomson, J. A., Mneney, E. E. and Oduor, R. O. (2016). Drought tolerance in transgenic tropical maize (*Zea mays* L.) by heterologous expression of peroxiredoxin2 gene-*XvPrx2*. *Afr. J. Biotechnol*. **15:** 1350–1362.

Sharma, P. I., Jha, A. B., Dubey, R. S. and Pessarakli, M. (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany*. **2012:** 26. doi: 10.1155/2012/217037.217037 .

Sharma, P. and Dubey, R. S. (2005). Drought induces oxidative stress and enhances the activities of antioxidant enzymes in growing rice seedlings. *Plant Growth Regul*. **46(3):** 209–221.

Shashidhar, H.E., Henry, A. and Hardy, B. (2012). Methodologies for root drought studies in rice. International Rice Research Institute. 65 p.

Shcherbakova, A. and Kacperska, A. (1983). Water stress injuries and tolerance as related to potassium efflux from winter rape hypocotyls. *Physiologia Plantarum*. **57:** 296–300.

Sheela, K. R. and Alexallder, V. T. (1995). Physiological response of rice varieties as influenced by soil moisture and seed hardening. *Ind. J. Plant Physiol*. **38 (3):** 269 – 271.

Shehab, G .G., Ahmed, O. K. and El-Beltagi, H. S.(2010). Effects of various chemical agents for alleviation of drought stress in rice plants (*Oryza sativa* L.). *Not Bot Hort Agrobot Cluj-Napoca*, **38(1):** 139–148.

Shi, L., Guo, M., Ye, N., Liu, Y., Liu, R., Xia, Y., Cui, S. and Zhang, J. (2015). Reduced ABA accumulation in the root system is caused by ABA exudation in upland rice (*Oryza sativa* L. var. Gaoshan1) and this enhanced drought adaptation. *Plant and Cell Physiology*. **56:** 951-964.

Shivachandran, R. and Kalarani, M.K. (2009). Transformation of elite rice cultivars ASD16 and IR64 with *cry2Ac* gene for resistance to rice lepidopteran pests. *Asian Journal of Bio Science.* **5(1):** 19-28.

Shivakumara, T. N., Sreevathsa, R., Dash, P.K., Sheshshayee, M.S., Papolu, P.K., Rao, U., Tuteja, N. and Udayakumar, M. (2017). Overexpression of Pea DNA Helicase 45 (PDH45) imparts tolerance to multiple abiotic stresses.

Siddique, M.R.B., Hamid, A. and Islam, M.S. (2001). Drought stress effects on water relations of wheat. *Bot. Bull. Acad. Sinica*. **41:** 35–39.

Sikdar, S.U., Zobayer, N., Nasrin, S. and Prodhan, S. H. (2015). *Agrobacterium* mediated PsCBL and PsCIPK gene transformation to enhance salt tolerance in indica rice (*O. sativa*). *In. vitro Cell. Dev. Biol. Plant.* **51:** 143-151.

Singh, A., Sengar, K. and Sengar, R. S. (2013). Gene regulation and biotechnology of drought tolerance in rice. *Int. J. Biotechnol. Bioeng. Res*. **4(6):** 547–552.

Singh, S., Gupta, A.K. and Kaur, N. (2012). Differential responses of antioxidative defence system to long–term field drought in wheat (*Triticum aestivum* L.) genotypes differing in drought tolerance. *Journal of Agronomy and Crop Science*. **198:** 185–195.

Singh, V.K., Mishra, A., Haque, I. and Jha, B. (2016). A novel transcription factor-like gene SbSDR1 acts as a molecular switch and confers salt and osmotic endurance to transgenic tobacco. *Sci Rep*. **6:** 31686.

Singha, L. D., Tuteja, N., Boro, D., Hazarika, N.G. and Singh, S. (2017). Heterologous expression of *PDH47* confers drought tolerance in *indica* rice. *Plant Cell Tissue and Organ Culture*. **130:** 577-589.

Sivamani, E., Bahieldin, A., Wraith, J. M., Al-Niemi, T., Dyer, W. E., Ho, T.H. D. and Qu, R. (2000). Improved biomass productivity and water use efficiency under water deficit conditions in transgenic wheat constitutively expressing the barley *HVA1* gene. *Plant Science*. **155:** 1-9.

Smimoff, N. (1993).The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytol.* **125:** 27-58.

Smith, S. and De Smet, I. (2012). Root system architecture: insights from *Arabidopsis* and cereal crops. *Philos. Trans. R. Soc. B. Biol. Sci.* **367:** 1441–1452. doi: 10.1098/rstb.2011.0234.

Subbarao, G.V., Nam, N.H., Chauhan, Y.S. and Johansen, C. (2000). Osmotic adjustment, water relations and carbohydrate remobilization in pigeonpea under water deficits. *J. Plant Physiol.* **157:** 651–659.

Sudhakar, P., Babitha, M., Latha, P., Prasanthi, L. and Reddy, P. V. (2006). Thermostability of cell membrane and photosynthesis in blackgram genotypes differing in heat tolerance. *J. Arid Legumes*. **3(2):** 11-16.

Sunitha, M., Srinath, T., Reddy, V.D. and Rao, K.V. (2017). Expression of cold and drought regulatory protein (CcCDR) of pigeonpea imparts enhanced tolerance to major abiotic stresses in transgenic rice plants. **245:** 1137 – 1148.

Szabados, L. and Savoure, A. (2009). Proline: a multifunctional amino acid. *Trends Plant Sci.* **15:** 89–97.

Takeda, S. and Matsuoka, M. (2008). Genetic approaches to crop improvement: responding to environmental and population changes. *Nature Reviews Genetics*. **9:** 444-457.

Tang, Y., Bao, X., Zhi, Y., Wu, Q., Guo, Y., Yin, X., Zeng, L., Li, J., Zhang, J., He, W., Liu, W., Wang, Q., Jia, C., Li, Z. and Liu, K. (2019). Overexpression of a MYB Family Gene, *OsMYB6*, Increases Drought and Salinity Stress Tolerance in Transgenic Rice. *Front. Plant Sci*. https://doi.org/10.3389/fpls.2019.00168.

Taylor, I.B., Burbidge, A. and Thompson, A.J. (2000). Control of abscisic acid synthesis. *Journal of Experimental Botany*. **51:** 1563-1575.

Taylor, N.J. and Fauquet, C.M. (2002). Microparticle bombardment as a tool in plant science and agricultural biotechnology. *DNA Cell Biol.* 21: 963-977.

Tran, T.N. and Sanan-Mishra, N. (2015). Effect of antibiotics on callus regeneration during transformation of IR64 rice. *Biotechnology Reports*. **7:** 143-149.

Tripathy, J.N., Zhang, J., Robin, S., Nguyen, T.T. and Nguyen, H.T. (2000). QTLS for cell-membrane stability mapped in rice (*Oryza sativa* L.) under drought stress. *Theor. Appl. Genet.* **100:** 1197-1202.

Turkan, I., Bor, M., Ozdemir, F. and Koca, H. (2005). Differential responses of lipid peroxidation and antioxidants in the leaves of drought tolerant *P. acutifolius Gray* and drought-sensitive *P. vulgaris L.* subjected to polyethylene glycol-mediated water stress. *Plant Sci.* **169:** 223-231.

Turner, N.C. (1986). Crop water deficits: a decade of progress. *Advances in Agronomy.* **39:** 2-51.

Tuteja, N. (2003). Plant DNA helicases: the long unwinding road. *J. Exp. Bot.* **54:** 2201-2214.

Tuteja, N., Gill, S.S. and Tuteja, R. (2012). Helicases in Improving Abiotic Stress Tolerance in Crop Plants. Improving crop resistance to abiotic stress. Wiley-VCH Verlag GmbH and Co, KGaA, Germany, pp 433–445.

Tuteja, N., Sahoo, R.K., Garg, B. and Tuteja, R. (2013). OsSUV3 dual helicase functions in salinity stress tolerance by maintaining photosynthesis and antioxidant machinery in rice (*Oryza sativa* L. cv. IR64). *The Plant Journal*. **76:** 115–127.

Tuteja, N. and Tuteja, R. (2004a). Prokaryotic and eukaryotic DNA helicases. Essential molecular motor proteins for cellular machinery. *Eur. J. Biochem*. 271: 1835–1848.

Tuteja, N. and Tuteja, R. (2004b). Unraveling DNA helicases. Motif, structure, mechanism and function. *Eur. J. Biochem*. 271: 1849–1863.

Tyagi, A.K., Mohanty, A., Bajaj, S., Chaudhury, A. and Maheshwari, S.C. (1999). Transgenic Rice: A Valuable Monocot System for Crop Improvement and Gene Research. *Critical Reviews in Biotechnology*. 19: 41-79.

Uga, Y., Sugimoto, K., Ogawa, S., Rane, J., Ishitani, M., Hara, N., Kitomi, Y., Inukai, Y., Ono, K., Kanno, N., Inoue, H., Takehisa, H., Motoyama, R., Nagamura, Y., Wu, J., Matsumoto, T., Takai,T., Okuno, K. and Yano, M. (2013). Control of root system architecture by DEEPER ROOTING 1 increases rice yield under drought conditions. *Nat. Genet.* **45:** 1097–1102.

Usman, M., Raheem, Z. F., Ahsan, T., Iqbal, A., Sarfaraz, Z. N. and Haq, Z. (2013). Morphological, physiological and biochemical attributes as indicators for drought tolerance in rice (*Oryza sativa* L.). *Eur J Biol Sci*. **5(1):** 23–28.

Vargas, W.A., Pontis, H.G. and Salerno, G.L. (2007). Differential expression of alkaline and neutral invertases in response to environmental stresses: characterization of an alkaline isoform as a stress response enzyme in wheat leaves. *Planta*. **226:** 1535–1545.

Varshney, R. (2011). Agricultural Biotechnology for crop improvement in a variable climate hope or hype? *Trends Plant Sci.* **16:** 363-371.

Vashisht, A. A. and Tuteja, N. (2005). Cold stress induced pea DNA helicase 47 is homologous to elF4A and inhibited by DNA interacting ligands. *Archives of Biochemistry & Biophysics.* **440:** 79-90.

Vashisht, A. A., Pradhan, A., Tuteja, N. and Tuteja, R. (2005). Cold and salinity induced bipolar pea DNA helicase 47 is involved in protein synthesis & stimulated by phospholyation with protein kinase C. *The Plant Journal.* **44:** 76-87.

Verbruggen, N. and Hermans, C. (2008). Proline accumulation in plants: A review. *Amino Acids*. **35(4):** 753–759.

Vinocur, B. and Altman, A. (2005). Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. *Curr Opin Biotechnol*. **16:** 123–132.

Vu, J.C.V, Gesch, R.W., Allen, L.H., Boote, K.J. and **Bowes, G. (**1999). CO_2 enrichment delays a rapid, drought- induced decrease in Rubisco small subunit transcript abundance. *Journal of Plant Physiology*. **155:** 139–142.

Wahid, A., Gelani, S., Ashraf, M. and Foolad, M.R. (2007). Heat tolerance in plants: an overview. *Environ. Exp. Bot.* **61:** 199–223.

Wang , C., Liu, S., Dong, Y., Zhao, Y., Geng, A., Xia, X. and Yin, W. (2016). PdEPF1 regulates water-use efficiency and drought tolerance by modulating stomatal density in poplar. *Plant Biotechnology Journal*. **14:** 849 - 860.

Wang, P.T., Liu, H., Hua, H.J., Wang, L. and Song, C.P. (2011). A vacuole localized β glucosidase contributes to drought tolerance in Arabidopsis. *Chinese Science Bulletin.* **56:** 3538 - 3546.

Wang, W., Vinocur, B. and Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta*. **218:** 1-14.

Wang, Y., Wisniewski, M., Meilan, R., Webb, R. and Fuchigami, L. (2005). Overexpression of cytosolic ascorbate peroxidase in tomato (*Lycopersicon esculentum*) confers tolerance to chilling and salt stress. *J. Amer. Soc. Hort. Sci*. **130:** 167–173.

Wang, Z., Han, Q., Zi, Q., Lv, S., Qiu, D. and Zeng, H. (2017). Enhanced disease resistance and drought tolerance in transgenic rice plants overexpressing protein elicitors from *Magnaporthe oryzae*. *PLoS One*. **12(4):** e0175734.

Wang, F.Z., Wang, Q.B., Kwon, S.Y., Kwak, S.S. and Su, W.A. (2005). Enhanced drought tolerance of transgenic rice plants expressing a pea manganese superoxide dismutase. *Journal of Plant Physiology.* **162:** 465 - 472.

Widawsky, D.A. and O'Toole, J.C. (1990). Prioritizing the rice biotechnology, rice agenda for Eastern India. *The Rockefeller Foundation.* pp 86.

Wilmink, A. and Dons, J. J. M. (1993). Selective agents and marker genes for use in transformation of monocotyledonous plants. *Plant Mol. Biol.* **11:** 165-185.

Wilson, J.B. (1988). A review of evidence on the control of shoot: root ratio, in relation to models. *Ann. Bot.* **61:** 433–449.

Wind, J., Smeekens, S. and Hanson, J. (2010). Sucrose: metabolite and signaling molecule. *Phytochemistry*. **71:** 1610–1614.

Worku, T. (1995). Water-retention capability as a selection criteria for drought resistance in wheat. *Journal of Plant Nutrition*. **18:** 2105-2109.

Xing, H. T., Guo, P., Xia, X. L. and Yin, W. L. (2011). PdERECTA, a leucine –rich repeat receptor-like kinase of poplar, confers enhanced water use efficiency in *Arabidopsis*. *Planta*. **234:** 229-241.

Xiong, L. and Zhu, J.K. (2003). Regulation of abscisic acid biosynthesis. *Plant Physiology*. **133:** 29-36.

Xu, D.Q., Huang, J., Guo, S.Q., Yang, X., Bao, Y.M., Tang, H.J. and Zhang, H.S. (2008). Over expression of a TFIIIA-type zinc finger protein gene *ZFP252* enhances drought and salt tolerance in rice (*Oryza sativa* L.). *FEBS Lett.* **582:** 1037– 1043.

Xu, Z. S., Chen, M., Li, L. C. and Ma, Y. Z. (2011). Functions and application of the AP2/ERF transcription factor family in crop improvement. *J. Integr. Plant Biol*. **53:** 570–585.

Yang, P. M., Huang, Q. C., Qin, G. Y., Zhao, S. P. and Zhou, J. G. (2014). Different drought-stress responses in photosynthesis and reactive oxygen metabolism between autotetraploid and diploid rice. *Photosynthetica*. **52**(2): 193–202.

Yang, S.J., Vanderbeld, B., Wan, J.X. and Huang, Y.F. (2010). Narrowing down the targets: towards successful genetic engineering of drought-tolerant crops. *Mol. Plant*. **3:** 469–490.

Yang, X., Wang, B., Chen, L., Li, P. and Cao, C. (2019). The different influences of drought stress at the flowering stage on rice physiological traits, grain yield, and quality. *Scientific Reports*. **9:** 3742.

Yu, L. H., Chen, X., Wang, Z., Wang, S. M., Wang, Y. P., Zhu, Q. S., Li, S. G. and Xiang, C.B. (2013). Arabidopsis enhanced drought tolerance1/ HOMEODOMAIN GLABROUS11 confers drought tolerance in transgenic rice without yield penalty. *Plant Physiol*. **162:**1378-1391.

Yu, L. H., Wu, S. J., Peng, Y. S., Liu, R. N., Chen, X., Zhao, P., Xu, P., Zhu, J. B., Jiao, G.L., Pei, Y. and Xiang, C.B. (2016). Arabidopsis EDT1/HDG11 improves drought and salt tolerance in cotton and poplar and increases cotton yield in the field. *Plant Biotechnol. J*. **14:** 72–84.

Yue, B., Xue, W.Y., Xiong, L.Z., Yu, X.Q., Luo, L.J., Cui, K.H., Jin, D.M., Xing, Y.Z. and Zhang, Q.F. (2006). Genetic basis of drought resistance at reproductive stage in rice: separation of drought tolerance from drought avoidance. *Genetics*. **172:** 1213–1228.

Zhang, G., Zhang, M., Zhao, Z., Ren, Y., Li, Q. and Wang, W. (2017). Wheat *TaPUB1* modulates plant drought stress resistance by improving antioxidant capability. *Scientific Reports*. **7:** 7549.

Zhang, H.X., Hodson, J.N., Williams, J.P. and Blumwald, E. (2001). Engineering salt-tolerant Brassica plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. *PNAS USA*. **98:** 12832–12836.

Zhang, J. and Tardieu, F. (1996). Relative contribution of apices and mature tissues to ABA synthesis in droughted maize root systems. *Plant Cell Physiology*. **37:** 598-605.

Zhang, L., Zhang, L., Sun,J., Zhang, Z., Ren, H. and Sui, X. (2013). Rubisco gene expression and photosynthetic characteristics of cucumber seedlings in response to water deficit. *Scientia Horticulturae*. **161:** 81- 87.

Zhang, Y.L., Zhang, H.Z., Du, M.W., Li, W., Luo, H.H., Chow, W.S. and Zhang, W.F. (2010). Leaf wilting movement can protect water-stressed cotton (*Gossypium hirsutum* L.) plants against photoinhibition of photosynthesis and maintain carbon assimilation in the field. *J Plant Biol*. **55(1):** 52 - 60.

Zhang, L., Xiao, S., Li, W., Feng, W., Li, J., Wu, Z., Gao, X., Liu, F. and Shao, M. (2011). Overexpression of a Harpin-encoding gene *hrf1* in rice enhances drought tolerance. *J. Exp. Bot.* 62(12): 4229 - 4238.

Zhao, B., Kondo, M., Maeda, M., Ozaki, Y. and Zhang, J. (2004). Water-use efficiency and carbon isotope discrimination in two cultivars of upland rice during different developmental stages under three water regimes. *Plant and Soil*. 261: 61-75.

Zheng, G., Fan, C., Dil, S., Wang, X., Xiang, C. and Pang,Y. (2017). Over-expression of Arabidopsis EDT1 gene confers drought tolerance in alfalfa (*Medicago sativa* L.). *Frontiers in Plant Science*. 8: 2125.

Zhou, W., Li, Y., Zhao, B.C., Ge, R.C., Shen, Y.Z., Wang, G. and Huang, Z.J. (2009). Overexpression of TaSTRG gene improves salt and drought tolerance in rice. *J. Plant Physiol.* **166:** 1660-1671.

Zhou, Y., Lam, H.M. and Zhang, J. (2007). Inhibition of photosynthesis and energy dissipation induced by water and high light stresses in rice. *J. Exp. Bot.* **58:** 1207–1217.

Zhu, B., Su, J., Chang, M., Verma, D. P.S., Fan, Y.L. and Wu, R. (1998). Overexpression of a D1 -pyrroline-5-carboxylate synthetase gene and analysis of tolerance to water- and salt-stress in transgenic rice. *Plant Science*. **139:** 41–48.

Zhu, M., Chen, G., Dong, T., Wang, L., Zhang, J., Zhao, Z. and Hu, Z. (2015). SlDEAD31, a putative DEAD-Box RNA helicase gene, regulates salt and drought tolerance and stress-related genes in tomato. *PLoSONE*. **10:** e0133849.

Zhu, Z. S., Xu, X. X., Cao, B. H., Chen, C. M., Chen, Q. H., Xiang, C. B.,Chen, G. and Lei, J. (2015). Pyramiding of AtEDT1/HDG11 and Cry2Aa2 into pepper (*Capsicum annuum* L.) enhances drought tolerance and insect resistance without yield decrease. *Plant Cell Tissue Org. Cult.* **120:** 919–932.

Zou, J., Liu, C., Liu, A., Zou, D. and Chen, X. (2012). Overexpression of OsHsp17.0 and OsHsp23.7 enhances drought and salt tolerance in rice. *J. Plant Physiol.* 169(6): 628-35.

Appendices…

APPENDIX I

A. Preparation of Alkaline lysis solutions

1. **Composition of Alkaline lysis solution I:**
   50 mM glucose
   25 mM Tris-Cl
   10 mM EDTA
2. **Composition of Alkaline lysis solution II:**
   0.2 N NaOH
   1% SDS
3. **Alkaline lysis solution III:**
   5 M potassium acetate
   Glacial acetic acid

B. RNase A preparation

The preparation of RNase A was done by dissolving 10mg of pancreatic Ribonuclease A (Sigma ,USA) in 1ml of 10mM Tris-HCl (pH -7.5) and 15 mM NaCl.The content was incubated over a boiling water bath at 100˚C for 15 mins and then, it was kept for cooling at room temperature and finally stored the aliquots of 20 ml at -20 ˚C.

APPENDIX II

Composition of MGL Medium

Chemical	For 1 Litre
Mannitol	5 g
L-Glutamic acid	1 g
KH_2PO_4	0.25 g
NaCl	0.1 g
$MgSO_4.7\ H_2O$	0.1 g
Tryptone	5 g
Yeast extract	2.5 g
Biotin	10µl (Stock : 0.1 mg/ml)

pH was adjusted to 7.0 using 1 N KOH and 1N HCl and autoclaved

APPENDIX III

A. Composition of 0.8% (wt/vol) agar plate (Hiei and Komari,2008)

1.6 g of agar (Difco) was added to 200ml of distilled water and finally, the mixture was autoclaved at 121˚C for 15 mins and cooled down to 50˚C, and then poured down 8ml of aliquots into Petri plates.

B. Composition of Hoagland's Solution (Plant Nutrient Solution; Hoagland and Arnon,1938)

	Chemical	**25 X stock/250ml**	**Quantity to be dispensed for 0.5 X solution per L**
	$MgSO_4.7H_2O$	12.683 g	
	KH_2PO_4	3.402 g	5 ml
	$CaNO_3$	29.528 g	5 ml
	$FeSO_4.7H_2O$	1.668 g	5 ml
	Na_2EDTA	1.753g	5 ml
	H_3BO_3	204.4 mg	
	$MnSO_4.H_2O$	151.0 mg	
		2.07 mg	
	KI	3.0 mg	5 ml
	Na_2MoO_4	24.9 mg	
	$CuSO_4$	2.26 mg	
	$CoCl.6H_2O$	3.0 mg	
	$ZnSO_4$		

APPENDIX IV

Composition of AB Plate (Hiei and Komari, 2008)

Reagents	For 1 L
Glucose	5 g
Bacto agar	4 g
20X AB salts	50ml of stock A + 50 ml of stock B
20 X AB buffer	50 ml
Antibiotic	Rifampicin (10mg/L) & Kanamycin (50 mg/L)

20 X AB salt: (Composition of stock A and stock B)

Composition of Stock A

Chemicals	For 1 L
NH_4Cl	20 g
$MgSO_4.7\ H_2O$	6 g
KCl	3 g
$FeSO_4.7H_2O$	50 mg

Composition of Stock B: 20 X $CaCl_2.7H_2O$

Chemicals	For 1 L
$CaCl_2.7H_2O$	265 mg

20 X AB Buffer

Chemicals	For 1 L
NaH_2PO_4	20 g
K_2HPO_4	60 g
Adjust the pH to 7.0 with 1N KOH and 1 N HCl	

APPENDIX V

Composition of AA infection Medium (Hiei and Komari, 2008)

Reagents	For 1 L
20X AA Major Salts	50 ml
20X $CaCl_2.2H_2O$	50 ml
100X FeEDTA	100ml
100X B5 minor salts	10 ml
100X B5 Vitamins	10 ml
10X AA amino acids	100ml
100 mM Acetosyringone	1 ml
Sucrose	20 g
Glucose	10 g
Vitamin assay casamino acids	0.5 g
Adjust the pH to 5.2 with 1 N KOH and 1N HCl	

STOCK A: Composition of 20 X AA Major salts

Chemicals	For 1 L
KCl	59 g
$MgSO_4.7H_2O$	10 g
NaH_2PO_4	3 g

STOCK B: Composition of 20 X $CaCl_2.2H_2O$

Chemicals	For 1 L
$CaCl_2.2H_2O$	3 g

100 x FeEDTA

Dissolve 2.78 g of $FeSO_4.7H_2O$ in 900ml of hot distilled water and add 3.73 g EDTA disodium salt. Cool to 25˚C and make upto the volume of 1 L.Store at 4˚C.

Composition of 100 X B5 Minor Salts

Chemicals	For 1 L
$MnSO_4.4H_2O$	1320 mg
$ZnSO4.7H_2O$	200 mg
$CuSO_4.5H_2O$	2.5 mg
$Na_2MoO_4.2H_2O$	25 mg
$CoCl_2.6H_2O$	2.5 mg
H_3BO_3	300 mg
KI	75 mg

Composition of 100 X B5 Vitamins

Chemicals	For 1 L
Myo-inositol	10 g
Thiamine HCl	1g
Pyridoxine HCl	100 mg
Nicotinic acid	100 mg

Composition of 10 X AA amino acids (pH- 5.8)

Chemicals	For 1 L
Glutamine	8.76 g
Aspartic acid	2.66 g
Arginine	1.74 g
Glycine	75 mg

APPENDIX VI

Composition of NB –As medium (Hiei and Komari, 2008)

Reagents	For 1L
10X N6 major salts	100 ml
10X $CaCl_2.2H_2O$	100 ml
100X FeEDTA	10 ml
100X B5 minor salts	10 ml
100X B5 vitamins	10 ml
2,4-D 100mg/L	20 ml
NAA 100mg/L	10 ml
6BA 100mg/L	10 ml
Sucrose	20 g
Glucose	10 g
Proline	0.5 g
Vitamin cassamino acid	0.5 g
Phytagel	4 g
100 mM acetosyringone	1 ml

10 X N6 Major Salts

Chemicals	For 1 L
KNO_3	28.3 g
$(NH_4)_{2.}SO_4$	4.63 g
$MgSO_4.7H_2O$	1.85 g
KH_2PO_4	4 g
$CaCl_{2.}2H_2O$	1.66 g

APPENDIX VII

Composition of CCMC medium (Hiei and Komari, 2008)

Reagents	For 1 L
10X CC Major salts	100 ml
10X $CaCl_2.2H_2O$	100 ml
100X FeEDTA	10 ml
100X CC Minor salts	10 ml
100X CC Vitamins	10 ml
2,4-D (100 mg/L)	20 ml
NAA (100 mg/L)	10 ml
6 BA (100 mg/L)	2 ml
Maltose	20 g
Mannitol	36 g
Proline	0.5 g
Vitamin cassamino acid	0.5 g
pH was adjusted to 5.8 with 1N KOH and 1 N HCl	
Phytagel	5 g
Cefotaxime (250 mg/L)	1 ml
Timentin	1 ml

10 X CC Major Salts

Chemicals	For 1 L
KNO_3	12.12 g
NH_4NO_3	6.4 g
$MgSO_4.7H_2O$	2.47 g
KH_2PO_4	1.36 g
$CaCl_2.2H_2O$	5.88 g

100 X CC Minor Salts

Chemicals	For 1 L
$MnSO_4.4H_2O$	1115 mg
$ZnSO_4.7H_2O$	576 mg
$CuSO_4.5H_2O$	2.5 mg
$Na_2MoO_4.2H_2O$	24 mg
$CoSO_4.7H_2O$	2.8 mg
H_3BO_3	310 mg
KI	83 mg

100 X CC Vitamins

Chemicals	For 1 L
Myo-inositol	9 g
Thiamine HCl	850 mg
Pyridoxine HCl	100 mg
Nicotinic acid	600 mg
Gylcine	200 mg

APPENDIX VIII

Composition of CCMCH30 medium (Hiei and Komari, 2008)

Reagents	For 1 L
10X CC Major salts	100 ml
10X $CaCl_2.2H_2O$	100 ml
100X FeEDTA	10 ml
100X CC Minor salts	10 ml
100X CC Vitamins	10 ml
2,4-D (100 mg/L)	20 ml
NAA (100 mg/L)	10 ml
6 BA (100 mg/L)	2 ml
Maltose	20 g
Mannitol	36 g
Proline	0.5 g
Vitamin cassamino acid	0.5 g
pH was adjusted to 5.8 with 1N KOH and 1 N HCl	
Phytagel	5 g
Cefotaxime (250 mg/L)	1 ml
Timentin	1 ml
Hygromycin B (30 mg/L)	1.5 ml

10X CC Major Salts

Chemicals	For 1 L
KNO_3	12.12 g
NH_4NO_3	6.4 g
$MgSO_4.7H_2O$	2.47 g
KH_2PO_4	1.36 g
$CaCl_2.2H_2O$	5.88 g

100X CC Minor Salts

Chemicals	For 1 L
$MnSO_4.4H_2O$	1115 mg
$ZnSO_4.7H_2O$	576 mg
$CuSO_4.5H_2O$	2.5 mg
$Na_2MoO_4.2H_2O$	24 mg
$CoSO_4.7H_2O$	2.8 mg
H_3BO_3	310 mg
KI	83 mg

100X CC Vitamins

Chemicals	For 1 L
Myo-inositol	9 g
Thiamine HCl	850 mg
Pyridoxine HCl	100 mg
Nicotinic acid	600 mg
Gylcine	200 mg

APPENDIX IX

Composition of NBPRCH30 medium (Hiei and Komari, 2008)

Reagents	For 1 L
10X N6 Major salts	100 ml
100X FeEDTA	10 ml
100X B5 Minor salts	10 ml
100X B5 Vitamins	10 ml
10X 2,4-D (100mg/L)	2 ml
10X NAA (100mg/L)	1 ml
10X 6BA (100 mg/L)	1 ml
Maltose	30 g
Proline	0.5 g
Vitamin cassamino acid	0.5 g

pH was adjusted to 5.8 with 1N KOH and 1N HCl

Phytagel	5 g
Gln (30 g/L)	10 ml
Cefotaxime (250 g/L)	1 ml
Hygromycin B (30 g/L)	1 ml

APPENDIX X

Composition of RNMH30 medium (Hiei and Komari, 2008)

Reagents	For 1 L
10X N6 major salts	100 ml
100X FeEDTA	10 ml
100X B5 minor salts	10 ml
100 X B5 Vitamins	10 ml
100 mg/L NAA (10 X)	1 ml
100 mg/L 6BA (10X)	0.5 ml
Maltose	30 g
Proline	0.3 g
Vitamin assay cassamino acid	0.3 g
The pH was adjusted to 5.8 with 1N KOH and 1N HCl	
Phytagel	5 g
Autoclave and cool to 50˚C	
Hygromycin B	0.6 ml

APPENDIX XI

Composition of 1X Transfer Buffer

COMPOSITION	For 1 L
Tris Base	3.0285 g
Glycine	14.26 g
20% Methanol	200 ml

APPENDIX XII

COMPOSITION OF TBS (as a Blocking Buffer)

COMPOSITION	For 1 L
Tris Base	6.057 g
NaCl	8.76 g