IDENTIFYING THE PHYSIOLOGICAL AND MOLECULAR BASIS OF PROLONGED (~3 WEEKS) SUBMERGENCE TOLERANCE IN RICE

M.Sc. (Ag.) THESIS

by

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COLLEGE OF AGRICULTURE

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IDENTIFYING THE PHYSIOLOGICAL AND MOLECULAR BASIS OF PROLONGED (~3 WEEKS) SUBMERGENCE TOLERANCE IN RICE

Thesis

Submitted to the

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Biswaranjan Das

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CERTIFICATE-I

This is to certify that the thesis entitled **"Identifying the physiological and molecular basis of prolonged (~3 weeks) submergence tolerance in rice"** submitted in partial fulfillment of the degree of **Master of Science in Agriculture** of the Indira Gandhi Krishi Vishwavidyalaya, Raipur, is a record of the bonafide research work carried out by **Biswaranjan Das** under my/our guidance and supervision. The subject of the thesis has been approved by Student's Advisory Committee and Director of Instructions.

No part of the thesis has been submitted for any other degree or diploma or certificate course. All the assistance and help received during the course of the investigations have been duly acknowledged.

Date: 18/07/19

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CERTIFICATE – II

This is to certify that the thesis entitled **"Identifying the physiological and molecular basis of prolonged (~3 weeks) submergence tolerance in rice"** submitted by **Biswaranjan Das** to the Indira Gandhi Krishi Vishwavidyalaya, Raipur, in partial fulfillment of the requirements for the degree of **Master of Science in Agriculture** in the Department of Plant Physiology, Agricultural Biochemistry, Medicinal and Aromatic Plants has been approved by the external examiner and Student's Advisory Committee after oral examination.

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Head of the Department

Faculty Dean

Approved/Not approved

Director of Instructions

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et. al.	And others
Cm	Centimetre
^{0}C	Degree Celsius
e.g.	For example
G	Gram
Hr.	Hours
M	Meter
Min	Minute
viz.	Namely
µm	Micrometer
µgm	Microgram
M ha.	Million hacter
no.	Number
%	Percentage
Q	Quintal
sec	Second
i.e.	That is
H_2O	Water

LIST OF ABBREVIATIONS

ADH	Alcohol Dehydrogenase
CAT	Catalase
c DNA	Complementary Deoxyribonucleic Acid
CI	Chloroform Isoamyl Alcohol
CRD	Complete Randomized Design
DNA	Deoxyribonucleic Acid
DW	Dry Weight
EA	Elongation Ability
EtBr	Ethidium Bromide
EDTA	Ethylene Diamine tetraacetic Acid
ERF	Ethylene Responsive Factor
FAOSTAT	Food and Agriculture Organization
LGF	Leaf Gas Film
NSC	Non Structural Carbohydrate
OD	Optical Density
Os	*Oryza sativa*
PDC	Pyruvate Decarboxylase
PCI	Phenol Chloroform Isoamyl Alcohol
RT-PCR	Real Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
SK	Snorkel
SOD	Superoxide Dismutase
SSR	Simple sequence repeat
SUB 1	SUBMERGENCE 1
QTL	Quantitative Trait Loci

THESIS ABSTRACT

a)	Title of the Thesis:	Identifying the physiological and molecular basis of prolonged (~3 weeks) submergence tolerance in rice.
b)	Full name of the Student	Biswaranjan Das
c)	Major Subject:	Plant Physiology
d)	Name and Address of the major advisor:	Dr.Pratibha Katiyar, Professor, Department of Plant Physiology, Agricultural Biochemistry, Medicinal and Aromatic Plants ,COA, IGKV, Raipur, Chhattisgarh.
e)	Degree to be Awarded:	M.Sc.(Ag)

P. Katiyar
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Signature of Student

Date: 18.07.2019

Signature of Head of Department

ABSTRACT

Rice is one of the major staple food crop in the world. It ensures livelihood security of the millions of people around the world, especially in economically weaker section of peoples of Asia.Though rice is a high water requiring crop, but excess water in the form of flash flood leads to severe damage to growth and productivity of rice. Due to increasing threat of climate change and erratic rainfall, the events of flash flood increased significantly leading to waterlogging conditions continuously for 2-3 weeks.The present study was conducted at National Rice Research Institute (NRRI), Cuttack, Odisha in collaboration with department of plant physiology,IGKV, Raipur to investigate the physiological strategies as well as the molecular factor(s) associated with prolonged (~3 weeks) submergence tolerance in the unique genotypes like AC42088, AC42087 and AC1303, which were having superior submergence

tolerance ability beyond 2 weeks. Submergence stress of different durations *viz*. 10 days, 14 days, 18 days and 22 days showed differential survival ability in the studied genotypes. Both FR13A and Swarna-Sub1 showed significant reduction in survival rate beyond two weeks of stress. At the end of three weeks of submergence, AC42088 was having highest survival, followed by AC42087 and AC1303. Higher survival in these genotype was supported by very low increase in plant height and elongation ability.Leaf gas film and tissue porosity was done for 7 days. AC1303 was found to possess highest thickness of leaf gas film 29.39 µm, followed by AC42088 and they could retain the gas film up to 8 days of submergence, which is longer than FR13A or Swarna-Sub1. Although highest tissue porosity (51%) was observed in FR13A, but both AC1303 and AC42088 were having ~50% tissue porosity under normal condition. The leaf surface was found to be hydrophobic in all the genotypes under control condition, but the contact angle was much higher in AC1303, AC42088 and AC42087 (>125°) as compared to Swarna (116°) or IR42 (112°). The underwater retention of hydrophobicity was higher in AC1303 as compared to FR13A. Similarly, the epicuticular wax content was highest in AC1303 (21.61 µgm). We found a comparatively slower breakdown of carbohydrate and chlorophyll degradation in all the three superior genotype, although there was not much difference between the leaf starch content of FR13A and these genotypes. The expression analysis of *SUB1A* gene showed that massive induction of expression was observed in FR13A, but it was only ~135-fold in susceptible genotype IR42. Surprisingly, the expression of *SUB1A* gene was intermediate in AC1303, a genotype having better prolonged submergence tolerance ability than FR13A, which indicated that there might some other factors apart from *SUB1* QTL in these genotypes contributing to their exceptional submergence tolerance behavior.

शोध सारांश

अ.	शोध शीर्षक	"चावल में लम्बे समय तक (~3 सप्ताह) जल मग्नता और सहिष्णुता के शारीरिक और आणविक आधार की पहचान करना"
ब.	विद्यार्थी का पूरा नाम	बिश्वरंजन दास
स.	मुख्य विषय	पादप कार्यिकी
द.	मुख्य परामर्शदाता का नाम एवं पता	डॉ. प्रतिभा कटियार, प्रोफेसर पादप कार्यिकी, कृषि जैव रसायन, औषधी एवं सुगंधित पौध विभाग कृषि महाविद्यालय, रायपुर, इं.गा.कृ.वि. रायपुर (छ.ग.)
इ.	उपाधि	स्नातकोत्तर (कृषि),

P. Katiyar
मुख्य परामर्शदाता के हस्ताक्षर

Biswaranjan Das
विद्यार्थी का हस्ताक्षर

दिनांक 18.07.2019

विभागाध्यक्ष के हस्ताक्षर

शोध सारांश

धान की फसल पानी की अधिकता मांगती है, परंतु अधिक पानी अचानक आई बाढ़ इसकी वृद्धि एवं उत्पादकता को नुकसान पहुंचाती है जलवायु परिवर्तन में मुख्यतः अनियमित वर्षा अचानक आई बाढ़ तथा जलभराव यदि लगातार 2–3 हफ्तों तक होता है तो यह धान की उत्पादकता के लिए खतरा हैं। इसी संदर्भ में यह अनुसंधान कार्य राष्ट्रीय धान अनुसंधान संस्थान, कटक उड़ीसा तथा इंदिरा गांधी कृषि विश्वविद्यालय, रायपुर के पादप कार्यिकी विभाग द्वारा समन्वय रूप में किया गया जिसमें धान की कार्य की रणनीति एवं आण्विक कार्य के द्वारा 3 हफ्तों से अधिक जलभराव रहने पर धान की 8 किस्मों की सहनशीलता परखने हेतु किया गया जिसमें से चार किस्मों को चेक के रूप में उपयोग किया गया, इस अध्ययन में 10,14,18 तथा 22 दिनों तक लगातार पानी का भराव करने के पश्चात् पौधों की अस्तित्व की क्षमता को मापा गया। FR13A तथा Swarna Sub 1 में दो हफ्तों से अधिक जल भराव में इसके अस्तित्व को खतरा हो जाता है जबकि AC42088 में यह क्षमता अधिकतम 3 हफ्तों तक दिखाई देती है, साथ ही यह कमशः AC42087 तथा AC1303 में भी दिखाई देती हैं जिसका संबंध पौधों की लंबाई में कम बढ़ने की क्षमता पाया गया इसके अलावा लीफ गैस फिल्म लेयर तथा ऊतक छिद्र के अध्ययन से यह ज्ञात हुआ कि AC1303 किस्म में लीफ गैस फिल्म लेयर की मोटाई अधिकतम 29.39μm थी जो 8 दिनों तक लगातार पानी के भराव में भी बनी रहती है, जो कि Swarna Sub 1 तथा FR13A (चेक) की तुलना में अधिक पाई गईं जबकि ऊतक छिद्र की क्षमता चेक किस्मों की तुलना में AC42088 तथा AC1303 में कम पाई गई। पत्तियों की जल प्रतिरोधक क्षमता को पानी के संपर्क कोण द्वारा मापा गया तथा यह ज्ञात हुआ AC42087 में यह >125 अधिक रहा जो कि चेक Swarna (116) तथा IR42 (112) की तुलना में अधिक पाया गया, साथ ही जलभराव की स्थिति में भी यह जल प्रतिरोधक क्षमता तथा पत्तियों में मोम की मात्रा 21.61 μg AC1303 में चेक की तुलना में अधिक पाई गई। साथ ही यह भी ज्ञात हुआ कि पर्णहरित तथा स्टार्च का विघटन इस सहनशीलता के लिए उपयोगी हैं। जलभराव के तीसरे दिन जीनों के व्यवहार का अध्ययन किया गया तथा यह ज्ञात हुआ जीन Sub 1 की अभिव्यक्ति FR13A में अधिक है यह जलभराव संवेदनशील किस्म FR42 से कम है, जबकि यह जीन AC1303 में मध्यम तरह की अभिव्यक्ति दे रहा है जो कि चेक की तुलना में अलग है।

CHAPTER I
INTRODUCTION

Rice (*Oryza sativa* L.) is the staple food in 39 countries of the world; it is one of the principal crops in humid and sub-humid Asia.It is classified primarily as a tropical and sub-tropical crop, cultivated as far as 49° N to 35° S, and from mean sea level to an altitude of nearly 3,000 meters. It provides approximately 32−59% of the dietary energy and 25−44% of the dietary protein consumed by the people of south and south east Asia. It also makes a significant contribution to the dietary energy and dietary protein consumed by the rice-eating population of Africa and Latin America. In India, rice is the staple food and cultivated in about 43.5 M ha area though having relatively low average productivity.

More than 50% of rice growing area in our country is adversely affected by different abiotic stresses like drought, flood and salinity which contribute to significant limitation for agricultural production and yield loss (Mahajan and Tuteja 2005). The present and anticipated global food demand pushes us for increasing crop production by means of enhanced productivity, particularly in less favorable rainfed lands. The new challenge of climate change will require resilience of rice production systems which can withstand multiple abiotic stresses coming simultaneously or in tandem during the cropping season. Breeding high yielding popular rice varieties with inbuilt tolerance to abiotic and biotic stresses offers a sustainable and remunerative option to improve rice profitability. Rain-fed conditions in Asia are quite complex where multiple stresses frequently prevail and even follow in quick succession within a single cropping period (Reddy et al. 2009). Among different environmental constraints flash flood or submergence stress is one of the major catastrophic events that detrimentally affects plant survival and crop yield particularly in low-lying rice growing ecologies.

Complete submergence due to flooding is most prevalent in the low-lying rice growing areas of the world. Crop loss due to excess water and water logging is also considerably high in such regions. A total of 22 million hectares of rice-

growing area is adversely affected by flash flooding, half of which is in eastern India.(Manoranjan et al. 2017). Although rice is a water loving crop and require considerable amount of water for normal growth but overabundance of water during submergence or waterlogging is destructive and fatal. Globally, water stagnation due to floods wasthe reason of almost 66% of all harm and misfortune to crops in the period of 2006 and 2016, with an estimated lossinbillions of dollars (Food and Agriculture Organization of the United Nations [FAO], 2017)

Submergence is a type of flooding stress where the entire plant canopy is fully immersed in water (complete submergence) or at least part of the shoot terminal is maintained above the water surface (partial submergence). Broadly, plants employ two strategies to cope up with the anoxia, arise due to submerged conditions. One is known as quiescence or low-oxygen quiescence syndrome, where plants exhibit very limited growth and elongation duringthe period of submergence stress (Colmer and Voesenek. 2009) and the other is an escape strategy called low-oxygen escape syndrome, where the plants show faster elongation of internode to come out of water as quickly as possible(Bailey-Serres and Voesenek. 2009).In quiescence strategy, plants during submerged condition do not shows any growth to preservetheir carbohydrate reserve. But, the plants remain alive and resume its growth just after the standing water recedes by using their available carbohydrates reserve (Colmer and Voesenek. 2009).

Quantitative Trait Loci (QTL) examination and map based cloning uncovered that the SUBMERGENCE 1 (SUB 1) locus, encoding a variable bunch of two or three tandem-repeats of ETHYLENE RESPONSIVE FACTOR (ERF-VII), regulate the quiescence response. Although, both strategies are completely opposite in nature, but interestingly both are governedby ethylene response factors. During quiescence Sub-1A gene action represses ethylene production by suppressing ERF-VII, thereby imparting a metabolic pause in growth and elongation in these genotypes under submerged condition to preserve their available carbohydrate reserve (Colmer and Voesenek 2009).On the contrary, in escape strategy plants quickly elongate their internode length to come up of water

level. Rapid internode elongation in such case is again governed by higher ethylene production and increased response of ethylene response factors, a process mediated by the action of *SNORKEL1* (*SK1*) and *SNORKEL2* (*SK2*) QTLs(Bailey-Serres and Voesenek 2008; Colmer and Voesenek 2009).

FR13A is a flash flood tolerant variety contains '*Submergence 1*' locus on chromosome 9 [(Xu and Maxkill 1996).Xu et al. (2006)] discovered *SUB 1* locus with *SUB1A, SUB1B* and *SUB1C* alleles were up regulated by submergence. But the SUB1A locus, which was found to impart almost two weeks of submergence tolerance. Being a native landrace of coastal Odisha, FR13A (otherwise known as Dhalaputia) is a submergence tolerant genotype, but like many other landraces it lacks superior other agronomic attributes *viz*., high yield and desirable grain quality traits. Hence, to transfer such important tolerance traits to cultivated background several intermediate breeding lines viz. IR49830-7 was generated (Mackil 1993 Mishra 1996).Finally, in 2002, Swarna which is one of the mega rice variety in India was crossed with IR49830-7 to produce submerge tolerance rice cultivar Swarna-Sub 1 having the same submergence tolerant quiescence strategy during the period of stress. Till date, FR13A is the most sought after submergence tolerant donor in rice and over the years it was used quite extensively to transfer the *SUB1A* QTL to different mega rice varieties like IR64, Samba Mahsuri, Chierang, Savitri etc. But, there are a few more genotypes identified *viz*. Kalaputia, IC459744, IC464746, IC399488, C9285 etc. Which can show considerable submergence tolerance up to 2 weeks. These genotypes were also known to possess SUB1 locus in chromosome 9 and their mechanism of tolerance are also well worked out (Xu and Mackill 1996, Xu et al. 2000, Chen et al. 2002, Septiningsih et al. 2009, Manivong et al. 2014).

Unfortunately, most of our modern high yielding rice cultivars are sensitive to submergence stress and cannot withstand complete submergence for 3-4 days. Onset of submergence stress leads to severe yield loss due to high plant mortality, low tillering and low to moderate recovery capacity of these genotypes (Ismail et al. 2008; Singh et al. 2009, 2013). A quick decrease in the oxygen (O_2) diffusion rate (~10,000-fold slower) in floodwater contrasted and in air hinders respiration

prompting an energy deficiency. This is especially extreme when photosynthesis is constrained or completely paused, as a result of obstructed internal diffusion of CO_2 and limited availability of underwater radiation. This results in plant death either during submergence or after de-submergence (Jackson and Ram 2003, Bailey-Serres and Voesenek 2008, Licausi and Perata 2009).

Plants employ different survival strategies under submergence include reduced internode elongation as a result of lesser ethylene production due *SUB1A* gene action, slower rate of leaf senescence, lesser depletion of NSC (non-structural carbohydrate) etc. (Panda et al. 2012, Septiningsih et al. 2013, Singh S et al. 2014).Studies over the years pointed out these are the major physiological/ metabolic processes in plants governing submergence tolerance ability. Non-structural carbohydrate (NSC) reserve is the primary substrates for producing energy when plants are immersed under water. Complete submergence leads to fast utilization these reserves and a commencement of protein hydrolysis in submergence intolerant genotypes (Setter et al. 1987, Michael B. Jackson et al. 2003). An assessment of submergence-tolerant and submergence-sensitive rice revealed that the seedlings of tolerant landraces usually had 30–50% more NSC than submergence intolerant genotypes (Chaturvediet al. 1996, Sarkar 1998).The reserved NSCs are used at the time of submergence to provide energy to support regular metabolic processes (Sarkar et al. 1996).The content of NSC reserved inside the dry seed or in the shoots of 10-day-old seedlings prior to submergence was not found to be significantly higher in submergence-tolerant genotypes, yet these genotypes would in general lose less carbohydrate for energy production when submerged, and recuperate quicker after submergence (Mazaredo and Vergara 1982, Das et al. 2005). Besides the carbohydrate reserve, the submergence tolerant genotypes found to retain the chlorophyll in leaves for a longer period due to *SUB1*-mediated blocking ethylene responsiveness in these genotypes. Also, the reactive oxygen species (ROS) produced during submergence stress or just after de-submergence were comparatively lower in tolerant genotypes (Sarkar et al. 1997). Lesser ROS production coupled with higher enzymatic and non-enzymatic scavenging activities of cellular antioxidants *viz.* ascorbic acid, glutathione, phenolic compounds and antioxidant enzymes like superoxide dismutase. Catalase,

peroxidase was found in tolerant genotypes, in addition to their better chlorophyll maintenance ability (Ella et al., 2003).

Apart from these well-known factors, leaf hydrophobicity and leaf gas film thickness also play important role in submergence tolerance in rice (Pedersen et al. 2009, Winkel et al. 2013; Colmer et al. 2014). Some of the previous studies showed leaf gas film (LGF) thickness trait is not related to *SUB1A* QTL, the major source of submergence tolerance in rice (Winkel et al. 2014). Whereas there are also a few reports where it was shown this trait may have considerable association with *SUB1* QTL in rice (Guru et al. 2018, Kurokawa et al. 2018) showed that the LGF thick in rice is governed by the action of an epicuticular wax biosynthesis pathway gene *LGF1* (*Leaf Gas Film 1*), which help them to maintain under water leaf hydrophobicity. Leaf gas films act to facilitate underwater gas exchange and support underwater photosynthesis for initial few days of submergence. *LGF1* regulates biosynthesis of a C-30 essential alcohol, associatedwith the formation epicuticular wax coating required for leaf hydrophobicity and development of gas films on submerged leaves (Kurokawa et al. 2018). It was found that during submergence, if the leaf gas films is retained for a longer period, it improves the effective utilization of reserved carbohydrates.(Pedersen et al. 2009).

In the nature we are gifted with wide diversity of rice germplasms. Being a primary center of origin of rice, there are enough diverse rice genotypes naturally occurring in environmentally challenging areas of our country. Exploration and characterization of such germplasm can give us novel rice accessions with unique superior traits for stress tolerance and many more. Exploring these diverse gene pool for rice, preliminary work showed us that submergence tolerance beyond two weeks can be achievable in rice. A few genotypes were reported to possess prolonged duration of submergence stress (about 3 weeks), a trait which is not present in the most widely used submergence tolerant donor FR13A globally. Although, studies over the years had information enough on mechanism of submergence tolerance in genotypes like FR13A. But, the question remains whether, the mechanism of tolerance in these superior lines is

same that of the known donor of submergence tolerance like FR13A, Swarna-Sub1 or there may be some new mechanism, new regulation of tolerance strategy operating in these genotypes. As these genotypes can withstand even longer duration of stress (~3 weeks), where FR13A fails to survive, so it creates enough doubt that whether they follow a similar mechanism of FR13A or not. Moreover, if the cumulative and complementary action of different submergence tolerance strategies is important in this case, then we need to know which components play key role in imparting additional tolerance in these genotypes. So, the present study is hypothesized to investigate the physiologicalstrategies as well as the molecular factor(s) associated with prolonged (~3 weeks) submergence tolerance in the unique genotypes like AC42088,AC42087 and AC1303, which are having superior submergence tolerance ability beyond 2 weeks of stress with the following objectives.

Objectives:

1. Identify and evaluate rice genotype having more than two weeks of submergence tolerance.
2. Morphophysiological and biochemical characterization of leaf gas film related traits in rice genotype having more than two-week submergence tolerance.
3. Molecular characterizations for submergence tolerance of these rice genotypes using gene based marker.
4. Study the comparative expression profile of submergence related gene in the genotype having more than two week of submergence tolerance.

CHAPTER –II

REVIEW OF LITERATURE

Being one of the most important crops of the world, rice holds a special position in maintaining the optimum food security in most of the South-Asian countries like: India, Bangladesh, Myanmar etc. Singh et al. (2017), whereas, China and India are regarded as the two biggest rice producing countries in all over the world (FAOSTAT 2013).

Singh et al. (2017) reported that rice is developed generally in assorted agro-climatic conditions going from high elevations of the Himalayan slopes to the ocean shorelines of the subcontinent of India and South-Eastern Asia.

Singh et al. (2016) reported that growth of population, environmental change and soil pollution adversely affect the rice productivity in a greater extent.

Sheehy et al. (2008) reported that the high yield gap in rain fed regions is often influenced by drought, flooding and salinity affecting rice productivity up to a greater extent. In Asia, rice yield potential is fundamentally stagnated and need to be increased by 60% in order to satisfy the food requirement of the growing population.

Vikram et al. (2016) proved that breeding of rice genotypes with inbuilt resistance offer a monetarily feasible and manageable alternative to improve rice productivity. Due to the heterogeneity in rain fed biological system, a wide range of rice cultivars are being developed by the breeders.

In this section an endeavour has been made to survey the available literatures on identifying the physiological and molecular basis of prolonged (~3 weeks) submergence tolerance in rice (*Oryza sativa L.*).The accessible literatures have been grouped into following headings.

2.1 Submergence stress:

Catling (1992), Nishiuchi et al (2013) characterized submergence resistance as "the capacity of a rice plant to endure 10–14 days of complete submergence and re-establish its development after de-submergence".

According to Khush et. al (1984)**,**Georgina V. et al. (2014) submergence tolerance is an important trait of rice (*Oryza sativa* L.) in rain-fed lowland conditions. This trait is controlled by an important gene designated as *Sub1* and the adaptation arises from well-developed arenchyma tissue that facilitates oxygen diffusion through continuous air spaces from shoot to root and by avoiding the development of anoxia and hypoxia conditions in root. Although rice is well adapted to waterlogged conditions, but long time flooding can adversely affect plant growth and development. More than 16% of rice lands of the world in lowland and deep water rice areas are adversely affected by flooding due to complete submergence.

Adkins et al. (1990), Pedersen et al.(2014) conferred that the impact of complete submergence on development and improvement of rice crops is variable with respect to the age of the plants, as the young plants show lesser tolerance capacity than that of the older ones.

Jackson and Ram (2003) demonstrated that complete submergence causes a number of damaging effects to the plants, including leaf degeneration, loss of dry biomass and a propensity of lodging.

Ram et al. (2000) reported that gas diffusion phenomenon is the major limiting factor for plant growth and development in the flood-prone environment. This is because gas diffusion is 10^4 fold lesser in water as compared to air (Armstrong 1979).

Carbon assimilation is an important process for growth and maintenance of the plants under submergence. Indian cultivar FR13A, is a highly tolerant rice variety which can survive up to 2 weeks of complete submergence owing to a major Quantitative trait locus designated as *submergence1* (*SUB1*) near the centromere of chromosome 9 (Xu and Mackill 1996, Xu et al. 2000, Chen et al. 2002, Septiningsih et al. 2013, Manivong et al. 2014).

Septiningsih et al. 2009, Manivong et al. 2014, Singh et al. 2014 announced that for submergence resilience the background hereditary data was all around recorded from various studies about utilizing QTL mapping and map based cloning approaches.

2.2 Morphophysiological and biochemical basis of submergence tolerance in rice:

2.2.1 Physiological studies on submergence tolerance of seedlings

2.2.2 Plant height, Elongation ability and total dry matter

2.2.3 Characters of leaf and role of leaf gas film

2.2.4 Role of epicuticular wax and leaf hydrophobicity

2.2.5 Aerenchyma formation

2.2.6 Starch content

2.3 Molecular mechanism of submergence tolerance

2.3.1 Sub1 QTL and submergence tolerance

2.3.2 Submergence related markers

2.2 Morpho-physiological and biochemical basis of submergence tolerance in rice:

Ram et al. (2007) conducted an experiment where 21 days old seedlings of six rice genotypes having variable submergence tolerance were exposed to 10 days complete submergence. Different parameters, like: Elongation ability, total starch content, and the production of antioxidant enzymes like: superoxide dismutase and catalase. The outcomes revealed that submergence tolerant genotypes, such as: Swarna-Sub1, FR13A and NDR 9730018 had higher starch status and lower shoot extension during submergence stress, hence exhibit higher plant survival. Activity of Catalase and SOD before submergence was normal in each of the genotypes. However, a substantial increase in the same was observed after de-submergence. Comparatively better results were obtained in the tolerant genotypes than that of the susceptible ones. Thus, he concluded that, this can be associated with higher survival rates observed in tolerant genotypes potentially through relieving the adverse impacts of reactive oxygen species. Relatively lesser rate of shoot elongation, high sugar content and higher post

submergence SOD action could be conceivable physiological markers for screening of rice germplasm for submergence tolerance.

Anupam et al. (2017) examined twelve rice genotypes on the basis of the biochemical changes because of submergence stress. Two more rice genotypes, FR13A and IR42, were likewise included as submergence tolerant and intolerant checked varieties respectively. The observed parameters include: Total chlorophyll and total protein content, solvent and insoluble starch, amylase, invertase, pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH). FR13A, Khoda, Kumrogore, Kalaputia, Meghi and Bhashakalmi showed lower rate of chlorophyll degradation during submergence. Bhashakalmi showed the highest amount of protein content in the plants experiencing stress. In FR13A, Meghi, Khoda, Bhashakalmi, Swarna-sub1, total amount of soluble sugar content was found to be higher on the lower side of the plant. In Moule, Harmanona, Panibhasha, Narayankamini, presence of high amount of sugar processing proteins (amylase and invertase) can be related to their survivability under stress. Apart from FR13A, in Meghi and Khoda, a marked increase of ADH and PDC (chemicals of alcoholic maturation pathway)was observed, which is the characteristic of their better execution under submergence stress. From this general examination they concluded that Meghi, Khoda, Kumrogore, Bhashakalmi and Kalaputia are the best performing genotypes under flooding stress.

2.2.1 Physiological studies on submergence tolerance of seedlings:

Ram et al. (2007) carried out an experiment where 21 days old plants of six rice genotypes differing in submergence tolerance were subjected to 10 days complete submergence. They examined various parameters such as underwater shoot elongation, shoot carbohydrate status and the activity of anti-oxidant enzymes viz.7 superoxide dismutase and catalase. The results indicated that submergence tolerant varieties Swarna-Sub1, FR13A and NDR 9730018 had higher carbohydrate status and lower shoot elongation during submergence, consequently higher plant survival. Catalase and SOD activity before submergence was almost at par in all the genotypes but increased after de-submergence. Submergence tolerant genotypes had better SOD

activity as compared to the intolerant ones. So, they hypothesized that this can be correlated with higher survival rates observed in tolerant genotypes possibly through mitigating the adverse effects of reactive oxygen species. Low underwater shoot elongation, high shoot carbohydrate and higher post submergence SOD activity could be possible physiological markers for screening of rice germplasm for submergence tolerance.

2.2.2 Plant height, Elongation ability and total dry matter :

Dwivedi et al. (1993) proved that the genotypes vary essentially in percentage of elongation at different water levels. Moderately more elongation (31.5%) was recorded at 100 cm of depth for drifting and deep water genotypes.

Sarkar et al. (2011) studied various rice genotypes having variable response to submergence on the basis of noticeable damage, submerged prolongation and plant survival. The derived conclusions from his experiment revealed no significant increase in the plant heights in SUB1introgressed cultivars but interestingly exhibited higher survival rates as compared to the other genotypes.

Pradhan et al. (2015) inspected 90 low land rice cultivars of the eastern parts of India, gathered and screened for submergence and water logging resilience and further utilized for approving the effectiveness of molecular markers and their association for submergence resistance. Submergence resistance and elongation ability of the desired genotypes were estimated in submergence tanks by taking the tolerant and susceptible checks. The genotypes FR13A, Khoda, CR Dhan 300, Savitri-Sub1, IR64-Sub1, IC-568009 and IC-568842 showed high submergence tolerance, which might be utilized as a major factor in the future breeding programs.'Khoda' showed tolerance to submergence stress with moderate elongation ability for adaption. Boitalpakhia, Gayatri, Atiranga, Aghonibora, Chakaakhi, Moti, IC-567993 and IC-568921 had the both the characters of moderate elongation ability and moderate resistance to submergence. Both of these qualities are required for lowland cultivars of eastern India to survive under flash flooding and complete submergence conditions.

2.2.3 Characters of leaf and role of leaf gas film:

According to Pedersen et al. 2009, Pedersen and Colmer, 2012) rice (*Oryza sativa*), a lowland crop which grows in shallow standing water and feeds an expansive extent of the total population, frequently hold a slender layer of gas termed as "leaf gas film".

Winkel et al.2014 reported that upper layer of leaves of rice is hydrophobic in nature. The submerged leaves have the ability to retain gas films which decreases with respect to time resulting anoxia and decreased photosynthetic rates.

Yusuke et al. (2018) worked on a rice variety (Kinmaze),showing the characteristic presence of gas films on the leaf surface, where its mutant (dripping wet leaf 7, drp7) showed absence of gas films. He found the essential role of leaf gas films in maintaining the underwater net photosynthesis. Leaf Gas Film 1 (*LGF1*) was identified as the gene responsible for the development of leaf gas films. *LGF1* controls C30 alcohol union, which is vital for in exhaustible epicuticular wax platelets, leaf hydrophobicity and gas films on submerged leaves. This quality enhanced the rate of underwater net photosynthesis by 8.2 folds and hence becomes helpful in providing submergence tolerance. The discovery of *LGF1* gene provided an opportunity in revealing the more readily comprehend variation among rice genotypes for gas film maintenance capacity which ultimately discovered a new era for developing improved genotypes with higher rate of tolerance along with high yielding capacity by utilizing the concerned genes especially in the flood prone areas.

Raskin and Kende (1983) demonstrated that gaseous layers give a critical survival advantage even in the partially and fully submerged rice plants. They demonstrated that degradation in gaseous layers is directly related to the prompted loss of Chlorophyll and protein content contents in the partially or fully submerged plant parts, ultimately affecting the elongation ability.

Colmer and Pedersen (2008) reported the presence of gas films on submerged leaf surfaces. They stated the theories that leaf gas films upgrade CO2 take-up for net photosynthesis (PN) during light periods, and improve O2 take-up at the dark periods. At the point of disappearance of gas films, O2 take-up in darkness was at that point diffusion constrained at 20.6 k Pa though for certain leaves with gas films, O2 take-up

declined uniquely at approx. 4 kPa (COP(R) 54 mmolO2m-3. Gas films additionally improved CO2 take-up so that, during light periods, submerged PN was increased by 6 folds. In addition to this, gas films showed their importance in the processes like: transpiration and respiration processes as well.

Pedersen et al. (2009) exhibited that gas films on leaves of fully submerged plants allow the passage of O2 and CO2 during dark and light periods respectively. It was also noted that, at the point of disappearance of gas films, rate of underwater net photosynthesis reduced to just 20% of the rate with presence of gas films. Significant changes observed after 7 days of complete submergence in terms of substantial decrease in the tissue sugar level and elongation ability of the plants. Presence of gas films on the leaf surfaces interestingly elevated the internal aeration process even in the anoxia conditions. Inward air circulation of roots in anoxic medium, when shoots were in oxygen consuming floodwater in darkness or when in light, was improved impressively when leaf gas films were available. This in terms contributed towards submergence tolerance mechanism.

Winkel et al. (2014) detailed the relationship between gas film and underwater net photosynthesis (PN) as affected by genotype and submergence period. Four genotypes (FR13A, IR42, Swarna, and Swarna-Sub1) were submerged for 13 days in the field and presence of leaf gas films, chlorophyll content and underwater net photosynthesis were surveyed with respect to time of submergence. At high CO2 during the PN measure, all genotypes at first demonstrated high rates of submerged PN, and this rate was not influenced by time of submergence in FR13A. At greater CO2 concentration, submerged PN decreased in each of the four genotypes and this compared with loss of leaf gas films with time of submergence. FR13A held leaf gas films reasonably longer period of time as compared to other genotypes, yet gas film maintenance was not connected to *SUB1*. They stated that differing rice genotypes ought to be screened for gas film persistence at the period of submergence, as this attribute could conceivably increase the starch status and internal aeration inferable from expanded submerged PN, which adds to submergence tolerance in rice.

2.2.4 Role of epicuticular wax and leaf hydrophobicity:

Neinhuis & Barthlott, (1997), Koch & Barthlott, (2009) reported that hydrophobicity of leaf surface was quantified by contact angle measurements which showed that the drp7 mutant had become hydrophilic after 1 day of submergence whereas Kinmaze remained hydrophobic. Interestingly, leaves of Kinmaze possess a higher density of epicuticular wax platelets than those of the drp7 mutant and these wax platelets contribute to leaf hydrophobicity .Papillae density can also influence leaf hydrophobicity (Koch et al. 2009**)**

Barthlott and Neinhuis, (1997) found that Leaf hydrophobicity has been considered in detail for certain species (for example sacrosanct lotus, and it happens because of different macro-scale, micro-scale and nanostructures superficial leaves, papillae and epicuticular waxes, individually (Marmur, 2003, Koch and Barthlott, 2009) – despite of the fact that the epicuticular wax platelets are viewed as of specific significance (Koch and Barthlott, 2009, Herzog et al. 2017).

Samuels et al. 2008) reported that formation of epicuticular waxes includes fatty acyl-CoA stretching, the results of which are then catalyzed to essential alcohols by fatty acyl reductases, a procedure that in Arabidopsis is constrained by individuals from the CER gene family.

Zhang et al. (2016) found that in rice, *OsHSD1* encodes a hydroxyl steroid dehydrogenase (HSD) that influences leaf wax structure, which was recommended to happen by means of intuitive impacts of *OsHSD1* on articulation of CER qualities.

According to Zhang et al. (2016) leaf surface hydrophobicity was lost in an oshsd1 mutant, however the influence on leaf gas films, submerged photosynthesis and submergence resilience was not assessed. The significance of leaf hydrophobicity and gas films for submerged photosynthesis and submergence resistance of rice (Pedersen et al. 2009,Winkel et al. 2013, Colmer et al. 2014).

2.2.5 Aerenchyma formation:

Parlanti et al. (2011) and Steffens et al. (2012) concluded that at the time of flooding, aerenchyma arrangement occurs in internodes of rice plants by ethylene production

which in terms advancing development of superoxide radical, H_2O_2; while in lowland rice, this happens after flooding in leaf sheaths of rice cultivars.

Haque et al.(2010), Malik et al. (2003) and Rajhiet al.(2011) stated that for the most part, formation of aerenchyma takes 24-72 hours after the beginning of anaerobic treatment.

Parlanti et al. (2011) examined aerenchyma formation in FR13A, a *SUB1A* variety and in Arborio Precoce(AP) a non *SUB1A* variety showing fast shoot elongation when submerged. From this experiment he found that constitutive aerenchyma was present in both AP and FR13A varieties and it further increased under submergence. Submergence-induced ethylene synthesis was observed in AP only, FR13A did not show any increase in ethylene production. The results suggest that aerenchyma formation in FR13A is independent of ethylene signaling, and ROS appear to be important to regulate aerenchyma formation in this *SUB1A* variety.

2.2.6 Starch content:

Chaturvedi et al. (1996) Sarkar (1998) found that an assessment of submergence-tolerant and submergence-susceptible rice developed under normal conditions revealed that the seedlings of tolerant genotypes regularly had 30–50% more NSC as compared to the susceptible varieties.

Sarkar et al. (1996) reported that these NSC are used to provide energy at the time of submergence for growth and tolerance mechanism.

Ram et al. (2002) likewise observed that the measure of NSC contained inside the dry seed or in the shoots of 10-day-old seedlings before submergence was not really higher in submergence-tolerant genotypes; yet these genotypes will in general lose less sugar when submerged and recuperate quicker after submergence. (Mazaredo and Vergara 1982; Das et al. 2005)

Sarkar et al. (2004) recognized submergence tolerant genotypes and revealed the presence of large amounts of non-structural carbohydrate (NSC, starch and soluble sugars) and constrained submerged elongation is related to submergence tolerance in rice.

Panda et al. (2012) examined the non-structural starch (NSC) status and its catabolism along with elongation growth in rice cultivars either having or not having the *SUB1* quantitative attribute locus (QTL), for example Swarna and Swarna-Sub1.At the time of submergence, Swarna quickened the rate of stem and leaf elongation and expansion along with increase in NSC content. Interestingly, Swarna-Sub1 showed the presence of higher energy accumulation to that of non-submerged plants. Swarna-Sub1 demonstrated preferred use of sugar over that of Swarna by dynamic acceptance of alcohol dehydrogenase, starch phosphorylase and α-amylase compound action during submergence. Generally, submergence tolerance provided by the *SUB1* QTL is associated with better survival rate and usage of NSC than that of Swarna.

Das et al. (2005) assessed submergence tolerant and susceptible rice genotypes which revealed that seedlings of tolerant species have 30-50% more NSC content to that of the susceptible varieties.

2.3 Molecular mechanism of submergence tolerance:.

2.3.1 *SUB1* QTL and submergence tolerance:

Xu et al. (2006) reported three ERF genes, *SUB1A*, *SUB1 B*and *SUB1C,* were identified in the *SUB1* QTL region of chromosome 9.

Fukao and Bailey-Serres, (2008) reported that *SUB1A-1* is activated by ethylene accumulation at the time of submergence, which negatively affects the submerged prolongation by constraining ethylene-induced gibberellic acid (GA)-promoted development. *SUB1A-1* initiates accumulation of the GA signaling repressors, *SLR1* and *SLRL1*, which down regulates the expression of the GA-inducible genes, which ultimately ceases the development of the plants.

SUB1A- differentially upregulates traits related with brassinosteroid (BR) formation. Brassinosteroid then prompts CA2ox7, a GA catabolic chemical (Schmitz et al., 2013). Evidently, *SUB1* acts by diminishing the responsiveness of GA which is involved in cell extension and shoot development.

Shunsaku et al. (2012) found that rice (*Oryza sativa L.*) in contrast to different grains, can develop well in wet fields and is profoundly tolerant of abundance water stress, either from submergence or waterlogging.

Rice shows tolerance towards submergence stress by internal aeration and differential growth control mechanisms.Submergence1A (*SUB1A*) for quiescence strategy and *SNORKEL1 (SK1)* and *SNORKEL2 (SK2)* are involved in escape strategy of submergence tolerance. Then again, rice handles waterlogging stress by formation of lysigenous aerenchyma and a hindrance to radial O2 loss (ROL) in roots to supply O2 to the root tip.

Sarkar et al. (2009) made an analysis at the time of monsoon of 2005–07 under favourable rainfed lowlands and controlled submergence and revealed that under flash flooding, genotypes with *Sub1* endure complete submergence stress with turbid water for as long as 12 days, though genotypes without Sub1 failed to survive under the same conditions. Swarna-Sub1 gave higher grain yield than Swarna at all locales with a yield preferred position of up to 1.65 tons/ha (a normal of 0.81 tons/ha more than five destinations).The outcomes propose that rice genotypes with Sub1 have extraordinary potential for improving the efficiency of rain fed rice in terms of tolerance to flash flooding.

2.3.2 Submergence related markers:

Mackill et al. (1993) reported that producers at IRRI made crossed between the tolerant FR13A and high-yielding genotypes, for example, IR48 and IR36 in the mid- to late-1970s. Submergence tolerant lines with high productivity were devloped in the mid -1990s.

Frisch and Melchinger (2005) reported that, adequacy of MAS relies upon the accessibility of closely linked markers and additionally flanking markers for the target gene, the measure of the population, the quantity of crosses and the position and number of markers for background selection.

Quality based and intragenic *Sub1* DNA markers were created depending upon DNA sequences published by Xu et al. (2006) and accessible in the NCBI database.Primer3 was utilized to construct PCR primers and BLAST inquiry was utilized to find regions of closeness and to confirm the specificity of the markers for the *Sub1* target area.

Xu et al. (2006) found that for the yield improvement programs like high breed recognization, testing seed hereditary purity and linkage mapping etc., SSR markers

have been used. Despite of the fact that a single gene specifically Sub1A controls tolerance against submergence has been distinguished, the exchange of this quality through traditional breeding combined with MAS is still the best method to create submergence tolerant rice cultivars.

RM8300 is one of the nearest simple sequence repeat (SSR) markers downstream of *Sub1A*. This marker has been utilized as one of the recombinant markers in addition to various SSR markers situated at about 1.5–2.5 Mb or 6–10 cM upstream of RM8300. Also, exceedingly polymorphic SSR markers in the *Sub1* area were identified from the International Rice Genome Sequencing Project (IRGSP, Matsumoto et al. 2005).

S. Singh et al. IRRI stated that, during stress condition Sub1 lines were superior and there was no significant distinction under normal conditions. Preliminary field trials at different national agricultural research and extension systems (NARES) sites in India and Bangladesh confirmed these results (Sarkar et al. 2006).

Neeraja et al. (2007) found that a few of these markers are firmly connected and found upstream to the *Sub1* locus, for example RM23835 (5.5 Mb), RM23865 (6.2 Mb) and RM23869 (6.3 Mb), and are helpful for constraining the measure of the Sub1 introgression. The marker nearest to *Sub1* recently announced was RM23805 (4.5 Mb).

Matsumoto et al. 2005 reported that RM8300 is one of the closest simple sequence repeat (SSR) markers downstream of *Sub1A*. This marker has been used as one of the recombinant markers in combination with different SSR markers located at about 1·5–2·5 Mb or 6–10 cM upstream of RM8300. Additionally, highly polymorphic SSR markers in the *Sub1* region were identified from the International Rice Genome Sequencing Project (IRGSP).

According to Neeraja et al. (2007) the methods for creating submergence-tolerant genotypes of rice (*Oryza sativa L.*) including molecular investigations and assessment of submergence resistance, followed those recently depicted for Swarna-Sub1.

According to Collard and Mackill, (2008), MAB procedure was pursued for introducing the tolerant Sub1 allele into the mega genotypes by using flanking markers

utilized for recombinant determination to decrease the target introgression size and background markers used to choose for recurrent parent alleles.

Neeraja et al. 2007 found that most of the SSR markers are closely related and located upstream of the *Sub1* locus, e.g. RM23835 (5·5 Mb), RM23865 (6·2 Mb) and RM23869 (6·3 Mb), and are very useful for limiting the size of the *Sub1*introgression. The marker close to *Sub1* previously reported was RM23805 (4·5 Mb).

Linh et al. 2013, Usatov et al. (2015) found that introgression of the *Sub1A* locus into the high-yielding genotypes utilizing marker-assistance determination will prompt the improvement of new submergence tolerant lines.

CHAPTER-III

MATERIALS AND METHODS

The present study was carried out to understand and characterize the mechanism of tolerance in a few selected rice genotypes having prolonged submergence tolerance ability of about three weeks. To achieve the objectives mentioned earlier, a pot experiment was conducted in the *Kharif* season of 2018 and repeated in *Rabi* season of 2019 in the net house of Plant Physiology at ICAR-National Rice Research Institute, Cuttack, Odisha (20° 27' 45.07" N; 85° 52' 58.75" E). Different parameters and/or traits were estimated in laboratory and net house conditions during the entire course of the study. A brief description of the materials used and the techniques adopted during the present study are presented in this chapter.

3.1. Geographical orientation of the experimental site:

All the experiments were conducted in net houses of ICAR-National Rice Research Institute, Cuttack in the state of Odisha, one of the major rice growing state of our country. The institute is situated on the bank of the river Mahanadi in the deltaic plains of eastern India, 80 Km west from the Bay of Bengal. The latitude and longitude are 20.5° N and 86.0° E respectively with an altitude of 23.4 m above the mean sea level.

3.2 Plant materials:

Eight genotypes of rice (*Oryza sativa* L.) comprising of come cultivars and unique germplasms viz. *FR13A, Swarna, Swarna Sub-1, IR-42, AC1303, AC42088, AC42087* and *AC38575* were grown in earthen pots in the net house having normal atmospheric conditions. The details of genotypes used for the present study with their special attributes (if any) were presented in Table 3.1.

Table 3.1: Detail of studied genotypes and their origin and special character

Sl No.	Variety	Origin	Character
1.	Swarna Sub-1	IRRI, Philippines	Swarna Sub-1 can withstand floods of up to 12-14 days and have *SUB1* QTL introgressed from FR13A
2.	FR13A	NRRI, Odisha	Maturity duration (155 days), Flood tolerant variety having *SUB1* QTL
3.	IR42	IRRI, Philippines	Maturity duration (110-120 days) having yield potential more than 6 ton.
4.	Swarna	Odisha, India	Maturity duration (150-155 days) and have low Glycemic Index
5.	AC42088	India	Showed more than two-week submergence from primary screening.
6.	AC42087	India	Showed more than two-week submergence from primary screening.
7.	AC1303	India	Showed more than two-week submergence from primary screening.
8.	AC38575	India	Showed more than two-week submergence from primary screening.

3.3 Experimental Condition

Earthen pots of 15 cm height having 2.0 kg of Sun dried soil mixed with farm yard manure (3:1) were used for the present pot experiments. At the time of sowing, single superphosphate (P_2O_5) and muriate of potash (K_2O) were applied @ 192 and 80 mg per pot, respectively. Before sowing, seeds were kept in hot air oven by maintaining the temperature of 45˚C for 2 days in order to allow them to overcome dormancy. Seeds were placed in a moistened filter paper in the petri-plates and were allowed to germinate by providing adequate moisture. The germinated seedlings were transplanted in the earthen pots keeping 50 pots per genotype and allowed to grow under normal atmospheric condition with regular watering. Out of these, 10 pots were kept as control (where individual pots served as a replication) and the remaining 40 pots were subjected to submergence stress twenty-one days after transplanting. Submergence stress of different duration was imposed by removing 10 pots of each genotypes on 10^{th}, 14^{th}, 18^{th} and 22^{nd} day

and necessary observations were recorded. For imposition of submergence stress, cemented tanks of L×B×H: 2.2m×1.5m×1.2m and L×B×H: 2m×1.5×1.9m respectively, were used in which pots were completely immersed into 100 cm of standing water.

3.4 Climatic condition:

The experiment was conducted in Rabi season (January – April, 2019) where maximum temperature and minimum temperature ranging from 33.3°C to 20.3°C, respectively without any significant rainfall and relative humidity varies from 81.7% to 51.1%. Since the climatic condition considerably influence the growth, development and grain yield, the weather condition were recorded from meteorological records of National Rice Research Institute, Cuttack.

3.5 Observations recorded

3.5.1 Morpho-physiological Parameters

3.5.1.1 Plant height

Plant height (cm) from each pot was measured from the base of the stem to the tip of the longest leaf before submergence i.e. on day 0 and just after de-submergence i.e. 10^{th}, 14^{th}, 18^{th} and 22^{nd} day with the help of measuring scale and values were noted down.

3.5.1.2 Elongation ability

Extent of elongation of the plant shoot was measured as 'Elongation Ability (%)'. This was determined by subtracting plant height before submergence from the respective plant height after submergence and expressed in percentage (Sarkar *et al*. 2011).

Seedlings elongation ability = $\{H_2\text{-}H_1)/ H_1\} \times 100$ (cm/day)
H_1 = Initial height H_2 = Height after submergence

3.5.1.3 Survival percentage:

Survival percentage was calculated by scoring the number of survived plants before and after submergence under each genotype × treatment combination. Final plant survival was counted 5 days after de-submergence in case of each stress duration. The survival ability was calculated as

Percent of survival of the seedlings = (No. of survived seedlings after submergence/No. of seedling present before submergence) × 100.

3.5.1.4 Chlorophyll and Carotenoid Estimation:

A known amount of rice leaf tissue (50 mg) was suspended in 10 ml of 80% acetone, mixed well and kept at 4 ºC for 48 hours. Chlorophyll a, b and total chlorophyll and carotenoid contents were quantified in samples by reading the optical density of the extract at 470, 663 and 645 nm by using using UV-VIS spectrophotometer (UV-2600,SHIMADZU). The samples were analyzed in triplicates. The amount of chlorophyll and carotenoid was calculated according to Arnon (1949) and Lichtenthaler and Wellburn.

Chlorophyll a (mg/g) = 12.21 (OD_{663}) – 2.81(OD_{645}) × V/W x 1000

Chlorophyll b (mg/g) = 20.93 (OD_{645}) – 5.03(OD_{663}) × V/W x 1000

Total chlorophyll content (mg/g) = Chlorophyll a + Chlorophyll b

Carotenoid content (mg/g) = (1000 × OD_{470}) – ((3.27 × Chlorophyll) a + (104 × Chlorophyll b)) /229

Where OD = optical density

V = final volume of 80% acetone (10 ml)

W = weight of sample taken (0.05 gm)

3.5.1.5 Leaf gas film thickness

For measuring leaf gas film thickness, leaf samples were collected in daily basis starting from Day 0 of submergence stress and continued until complete disappearance of leaf gas film. The fresh weight of 10-cm-long leaf sections (excised from the mid portion of the leaf blades) was measured and width of the respective leaf segments was noted down. In order to keep the sections submerged in water, the sections were attached to a metal clamp of optimum weight. In order to keep the desired leaf sample and the clamp suspended in water, a wire was attached with a pan hook which kept fixed on analytical balance with the help of cello tapes. The entire experiment was carried out by keeping the frame in association with the leaf sections submerged in distilled water carried out in a preferably large container maintaining the optimum level of water. Weight of the submerged clamp (W_1), weight of the submerged clamp along with the leaf sections (W_2) were measured. In order to remove the

leaf gas film from both of the leaf surfaces (both adaxial and abaxial). The leaf sections were treated with Triton X-100 and the weight of the submerged triton treated leaf along with the clamp were noted down as W_3. Calculations were done based on the equations given by Raskin and Kande (1983): V_{air} = B/ density of water, where V_{air} = volume of air layers, B= buoyant force

B = $W_3 - W_2$, where W_3 = weight of the submerged clamp with triton-treated sections and W_2 = weight of the submerged clamp with the leaf sections.

V_{leaf} = B_{leaf} / density of water

3.5.1.6 Tissue porosity

Porosity (percentage gas spaces per unit tissue volume) of lamina was measured by determining tissue buoyancy before and after vacuum infiltration of the gas spaces with water (Raskin, 1983), using the equations as modified by Thomson et al. (1990). The buoyancy of the leaf-lamina (10cm) was measured by keeping it attached with a metallic frame which was suspended by a weighing balance (sensitivity up to 0.1mg) inside the de-ionized water after treating it with 0.1% Triton X-100. In order to ensure the complete removal of gases from leaf lamina tissues, the laminas were allowed to undergo the vacuum-infiltration process. This method was carried out for several times in order to ensure the complete removal of gases and the weight of the lamina was assessed for one more time. Proper precautions should be taken in order to ensure the avoidance of entry of external gases during the continuity of this process. The tissue porosity was measured by following the Archimedes' principle (Raskin, 1983); where % of porosity = (Volume of the gas in leaf/volume of the leaf × 100.

3.5.1.7 Leaf hydrophobicity:

Leaf hydrophobicity of each genotype was measured by excising a 10 cm long leaf clip and completely immersed in water in petri-plates. The contact angle of single water droplet formed on the leaf surface was measured in 0, 4, 24, 48 and 96 h after immersion. Hydrophobicity of the leaf cuticle was calculated by using the contact angle of small droplets of water. A contact angle of >90° indicate a hydrophobic surface and those >150° indicate super-hydrophobicity (Koch & Barthlott, 2009). Hydrophobicity of leaf blade segments of rice leaf was quantified

by measuring the contact angle of a 10µl water droplet on the adaxial side by using a pipette. The contact angle of each sample was calculated by averaging the values of three replications.

3.6 Biochemical analysis:

3.6.1 Estimation of Epicuticular wax:

Samples were collected periodically from submergence tank. The individual sample consisted of 7 rice leaf discs (5 cm each). Each sample was immersed in 15 ml of chloroform for 15 sec and redistilled for a few times. The extract was filtered and kept in a boiling water bath at 35 °C for complete evaporation of chloroform. After adding 5 ml of reagent ($K_2Cr_2O_7$), samples were placed in boiling water bath for 30 min. After cooling, 12 ml of deionized water was added to the samples and kept in room temperature for colour development. Then the optical density of the sample was measured at 590 nm by using UV-VIS spectrophotometer (UV-2600, SHIMADZU).

The principle of this method was based on the color change produced due to the reaction of wax with acidic $K_2Cr_2O_7$.The reagent was prepared by mixing 40 ml de-ionized water with 20 gm powdered potassium bicarbonate. The resulting solution was mixed vigorously with 1 liter concentrated sulfuric acid and heated (below boiling) until a clear solution was obtained.

Standard wax solutions were prepared from carnauba wax.

3.6.2 Estimation of Starch:

Starch content was determined by Anthrone Method (McCready *et al*, 1950). The dried leaf samples were powdered and a known amount of it was hydrolyzed by boiling with 10 ml of 1 N HCl in a glycerin bath at 112-115 ^{0}C for 30 minutes. The residue was repeatedly washed with distilled water until a negative test of starch by iodine was obtained. The extract was collected and the final volume was made up to 100 ml. An aliquot (0.5-1.0 ml) of the above extract was made to uniform volume of 2.5 ml with distilled water. It was then mixed thoroughly with 10 ml of freshly prepared anthrone reagent (100 mg of anthrone in 100 ml of chilled concentrated sulfuric acid) in a cold-water bath. The tubes were then kept in a boiling water bath for 15 min and cooled in running tap water.

Absorbance was measured at 620 nm by using UV-VIS spectrophotometer (UV-2600, SHIMADZU). One blank and two freshly prepared glucose standards were also included with each set of samples. Starch content was calculated by multiplying the glucose values by 0.9 (Pucher *et al*, 1948) and expressed in mg g^{-1} DW.

3.7 Molecular studies

3.7.1 Gene Expression Studies:

3.7.1.1. Total RNA Isolation:

RNA was isolated from rice leaf samples by using RNeasy Mini Kit (Qiagen, Germany) according to manufacturer's instructions. Samples were collected 3 days after submergence and dipped in 3 ml RNA Later (RNA Stabilization Reagent, QIAGEN, Germany). Before doing RNA extraction, tips, mortar-pestle, spatula etc. were dipped in DEPC treated water to reduce the risk of RNA being degraded by RNase followed by drying and then autoclaved. The working space was cleaned with 70% ethanol and RNase AWAY (SIGMA, Switzerland), which is a decontamination reagent for RNase.

100 mg of leaf sample was ground thoroughly with liquid nitrogen by using mortar and pestle. Powdered samples were transferred to 2 ml Eppendorf tubes and 450 µl RLT buffer (RLT+ β-mercaptoethanol) was added. A brief vortexing was done. A short (3 min) incubation at 56 ℃ was given for efficient disruption the tissue. The lysate was transferred to a Q1A shredder spin column placed in a 2 ml collection tube and centrifugation was allowed for 2 minutes at high speed. The supernatant was transferred carefully to a new microcentrifuge tube without disturbing the pellet. 0.5 volume of 96-100% ethanol was added to the lysate and was mixed immediately by pipetting. The sample was transferred to an RNase spin column placed in a 2 ml collection tube. The lid was closed gently and centrifugation was allowed for 1 minute at 10,000 rpm. The flow through was discarded and the spin column was reassembled with its collection tube. 350 µl of RW1 buffer was added and allowed for 2 min centrifugation. The flow through was discarded and the spin column was assembled with a new collection tube. 80 µl of DNase-I solution was added to the column and it was left undisturbed for 30 minutes. After that 350 µl of RW1 buffer was added to the column. 500 µl of RPE buffer was added to the

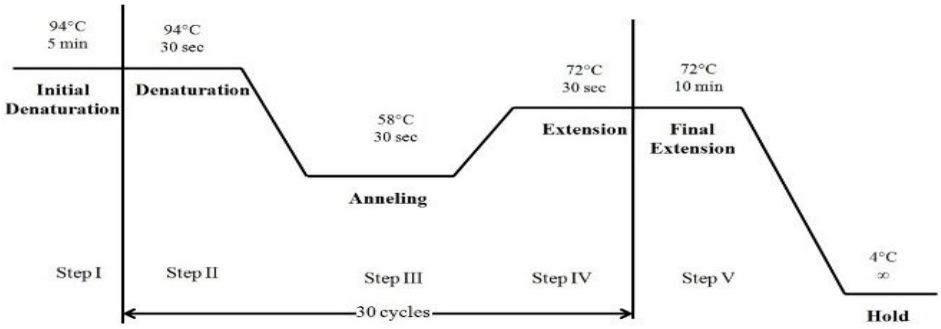
RNeasy spin column, the lid was closed gently and centrifuged for 2 minutes at 10,000 rpm to wash the spin column. Then the column was transferred to a new 2 ml collection tube and centrifuged for 1 minute at full speed. RNeasy spin column was placed in a new 1.5 ml collection tube. 30 µl of RNase-free water was added to the spin column and allowed for centrifugation for 1 minute at 10,000 rpm for elution of RNA. RNA was quantified by using Nanodrop (Eppendorf). RNA was checked on an agarose gel (1.2%).

3.7.1.2. Synthesis of single strand cDNA:

The cDNA synthesis describes the generation of complementary DNA (cDNA) from an RNA template by reverse transcription. Reverse transcriptase use an RNA template and a primer complementary to the RNA to direct the synthesis of the first strand cDNA**,** which can be used directly as a template for the polymerase chain reaction. cDNA synthesis was carried out using first strand cDNA synthesis kit (QIAGEN). gDNA wipe out buffer was added to a sterile RNase free microfuge tube. RNA sample and RNase free water was added to the tube. The mixture was incubated at 42˚ Celsius for 5 minutes. The cycle was paused and the mixture was immediately kept on ice. Then 6 µl of master mix (reverse transcriptase, reverse transcription buffer, reverse transcription primer mix) was added to each microfuge tube. It was allowed for incubation at 42˚ Celsius for 30 minutes. Then the reaction was allowed to stop at 95˚ Celsius for 3 minutes.

3.7.1.3. Checking of cDNA stock using 18s r RNA as a primer:

The reverse transcriptase PCR was carried out using all cDNA samples (about 100 ng) as template using β-actin as primer**.** The amplification was carried out following the following programme depicted in Fig 3.1. The PCR products were run on 3.5% (w/v) agarose gel and photograph was saved.



3.1: PCR amplification programme used for cDNA checking

3.7.1.4 Expression profiling of submergence related genes by q-PCR analysis:

Changes in transcript expression of submergence related genes were studied by Real-Time quantitative PCR. Real-time quantitative PCR was set using QuantiFast SYBR Green PCR reaction kit (Qiagen, USA). The reaction mixture includes about 100 ng of cDNA, 0.16 µM of primers and 12.5 µl of QuantiFast SYBR Green PCR mix. The volume of reaction was maintained to 25 µl by sterile nuclease free water. Reactions were run in Real-Time PCR System (Quant Studio 5, Applied Biosystems, Thermo Fisher Scientific, USA) and conditions were set as follow: 95 ℃-5 min for 1 cycle; 95 ℃-10 sec and 60 ℃-30 sec for 40 cycles. At the end of the PCR cycles, the products were put through a melt curve analysis to determine the specificity of amplification. The fold changes in transcript in stressed plants compared to healthy plants were analyzed by comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001; Schmittgen and Livak 2008). The *Os18s* gene was used as internal control to normalize the PCR reactions.

Table.3.2: Primers used for qPCR analysis in rice in response to submergence stress

Gene Name	Locus Id/Ac. No.	Forward Primer	Reverse Primer	Amplicon Size
18s	AK059783	ACATAGAAGGAGA	ACACTTCACCGGA	**65**
Rrna		AGAATGCACCCGC	CCATTCAA	
SUB1	DQ011598b	CGGCCTCATCACA	ATGTCCATGTCCA	**176**
A		ATCGGAG	TATGTCGTCG	
SUB1	LOC_Os09g1	GGACGCCACAACG	TGCACCAGAAGG	**139**
B	1480	AAGATGAAGAA	GAACATGGAAAC	
SUB1	LOC_Os09g1	ATACTCATCGAGT	TTAGCTCCAGAA	**90**
C	1460	GCTGCTCCGAC	GCGCATGTC	

3.7.2 Extraction of Genomic DNA:

3.7.2.1 Chemicals:

i. CTAB
ii. Isoamyl alcohol
iii. Chloroform
iv. β-mercaptoethanal
v. Ice cold absolute Ehanol
vi. Ice cold 70% ethanol
vii. RNase
viii. Sterile double distilled water

3.7.2.2 Procedure:

200 mg of leaves from germinated seeds were taken in mortar and grind to a fine paste in approximately 1000 µl of CTAB buffer. β-mercaptoethanal was added to the CTAB plant extract mixture and transferred to a microcentrifuge tube. The plant extract mixture was incubated for about 15 min at 65°C in a water bath. After incubation, the plant extract mixture was spinned at 8000 rpm for 5 mins to spin down the cell debris. The supernatant was transferred to clean microcentrifuge tubes. Add equal volume (25:24:1) of PCI (Phenol:Chloroform:Isoamyl Alcohol). Centrifused the tubes at 12000 rpm for 10 min. The supernatant was transferred to the fresh 1.5 ml tubes. To each tube equal volume (24:1) of CI (Chloroform:Isoamyl Alcohol) was added and mixed well by inversion. The tubes were spinned at 12000 rpm for 10 min. The upper aqueous phase was transferred to clean microcentrifuge tube. To each tube 500µl of ice cold absolute ethanol was added. The tubes were inverted slowly several times to precipitate the DNA. The tubes were placed for 1 hr at -20°C for precipitation of the DNA. Generally, the DNA can be seen to precipitate out of solution. To wash the DNA, the precipitate was transferred into a microcentrifuge tube containing 500µl of ice cold 70 % ethanol and slowly inverted the tube repeatedly. After washing, the DNA was spinned by centrifuging at 5000 rpm for 5 min to form a pellet. The supernatant was removed and DNA pellet was allowed to dry (approximately for 30 mins). DNA was re-suspended in sterile 50µl DNase free water. 4 µl of RNase (10µg/µl) was added to the water prior to dissolving the DNA to remove any RNA in the

preparation. After resuspension, the DNA was incubated at 65°C for 20 min to destroy DNases that may be present and stored at -20°C. Concentration of DNA was checked by Biospectrometer Fluorescence (Eppendorf).

3.7.3 Agarose gel Electrophoresis:

3.7.3.1 Chemicals:

i. Agarose powder
ii. 1X TBE (tris borate-EDTA)
iii. Ethidium bromide

3.7.3.2 Procedure:

All the samples DNA(0.8%) as well as PCR products (3%) were separated by electrophoresis in Agarose gel. Electrophoresis in Agarose gel was performed in 1X TBE (tris-borate- EDTA) running buffer (Sambrook et al. 1989). The gel was prepared by dissolving Agarose in 100 ml of 1XTBE buffer. The Agarose-TBE mixture was heated in a microwave oven until the Agarose was thoroughly melted. 2µl Ethidium bromide (from a stock solution of 10 µg/ml) was added and then mixed uniformly (Sambrook *et al.*, 1989). The melted agarose was then cooled to about 50°C and poured into a horizontal gel casting platform and the combs were inserted. The gel was poured making sure so that no air bubble were trapped underneath and in between the teeth of the comb. After the gel had been hardened, sufficient electrophoresis buffer (1×TBE) added to submerge the gel to a depth of at least 1mm. The gel was run by using power supply of 60 volt.

3.7.4 Validation of submergence tolerance genotypes with SSR markers:

26 SSR(Simple Sequence Repeats) markers which was specific to *SUB1* QTL was selected and was used for amplification. Primers were developed through primer 3 software and gramene database. For that DNA was isolated from the 8 genotypes and PCR amplification was performed with 26 primers. The product amplified run on the 3% agarose gel and picture was taken by gel documentation unit.

Table 3.3: Details of SSR marker :

SI. NO.	Oligo Name	5'<-----Sequence----->3' Forward Primer	5'<-----Sequence----->3' Reverse Primer
1	RM219	CGTCGGATGATGTAAAGCCT	CATATCGGCATTCGCCTG
2	RM 7175	ACAGTAAACGTGGTGCCTCC	AGAAGTAGCCTCGAGGACCC
3	RM 316	CTAGTTGGGCATACGATGGC	ACGCTTATATGTTACGTCAAC
4	RM 444	GCTCCACCTGCTTAAGCATC	TGAAGACCATGTTCTGCAGG
5	RM 464	AACGGGCACATTCTGTCTTC	TGGAAGACCTGATCGTTTCC
6	RM 7481	CGACCCAATATCTTTCTGCC	ATTGGTCGTGCTCAACAAG
7	RM 8303	AGGGGAGAGGACACACACAC	GGATCCTCCTGCAAAATCAA
8	RM 23877	TGCCACATGTTGAGAGTGATGC	TACGCAAGCCATGACAATTCG
9	RM23835	TTCCGCTGTTTCTCTTCTTGTGC	CTGGTTCTGCTGGTTCTGTAGTTGG
10	RM23865	TCATCCCATTCTCTTCCTCACC	CATACGGCCATACAAATGAACC
11	RM23869	GGCATATTCGTGTTGTCCTCACC	GCCACGCGTACCTGAGATATGG
12	RM23679	TCACAGCTTAGTGCATGTTGAGC	GATTCACCTGGCAATGAGAACG
13	RM23805	GAGACAGATGTGTACGGTTTGGTG	TTGACAAGGAAGGAGAAG
14	RM23915	GAGGATCCTTACCATCAAACTTCG	CCAAGAACCTGCATTCTTCAAGG
15	RM23958	CTACCACTGTTTCATTGTGTCTCG	GAATTGAAGGAGAAGCAGGAAGC
16	Sub1C173	AACGCCAAGACCAACTTCC	AGGAGGCTGTCCATCAGGT
17	ART3	TCTGAACCGGATCATCATTG	AGTTTGTCTCCATTCGAAGTCA
18	Sub1A203	CTTCTTGCTCAACGACAACG	AGGCTCCAGATGTCCATGTC
19	IYT1	TAGGGGCCCATGAGTACTTG	TCAGACAGCTAGCTCGCAAC
20	IYT3	GTTGATAACCGGAGGAGACG	GTAACCCGACTGGTCTCAGG
21	Sub1 AB 1	CATGTTCCATAGCCATCGACT	GAGCGAAGAGAGCTACCTGAA
22	Sub1 BC 1	CAATCGATGCGTGCTTCTT	CGCAACAAGGCAGAAAAATA
23	Sub1 BC2	AAAACAATGGTTCCATACGAGAC	GCCTATCAATGCGTGCTCTT
24	Sub1 BC 3	CATGGGTAAAATTGCCATCC	GCTTGAGGGTGAGTGGAGAG
25	ART5	CAGGGAAAGAGATGGTGGA	TTGGCCCTAGGTTGTTTCAG
26	RM8300	GCTAGTGCAGGGTTGACACA	CTCTGGCCGTTTCATGGTAT

3.8 Statistical Analyses:

All the data recorded were the mean values ± standard error (mean) of 3–10 independent replications. The experiment was conducted in two-factor completely randomized design and the data were subjected to two-way ANOVA as per the experimental design. The ANOVA found significant for treatment × genotype interaction at 5% level of significance.

CHAPTER-IV

RESULT AND DISCUSSION

A net house experiment was conducted during the *Rabi* season of 2019 in a factorial Completely Randomized Design (CRD) to study the physiological,biochemical and molecular basis of prolonged submergence tolerance in rice beyond two weeks.For this study was conducted under the topic **"Identifying the physiological and molecular basis of prolonged (~3 weeks) submergence tolerance inrice".**Different physiological, biochemical and molecular parameters were investigated during the course of the study. The results obtained from the present study have been presented under different sub-headings of the chapter and shown in appropriate tables and figures along with statistical interpretations of the data and adequately discussed in the light of already existing knowledge in the present field.

4.1: Morpho-physiological Parameters

4.1.1 Survival percentage:

The results from the present study showed that different durations of the complete submergence had differential impact on survival rate of the studied genotypes (Fig.4.1).At the end of three weeks of submergence. It was found that the genotype AC42088 was having highest survival rate of 83%, followed by AC42087 (66%) and AC1303 (50%). All the three genotypes performed significantly better than the known submergence tolerant check FR13A, which showed only 33% survival rate after three weeks of continuous submergence. Up to 14^{th} day of submergence stress, these three genotypes (AC42088, AC42087 and AC1303) and FR13A all showed 100% survival, where as it was little less in Swarna-Sub 1 (83%). Although, the genotype AC38575 showed slightly better survival than our susceptible check Swarna and IR42 at 10^{th} day of submergence stress, but eventually all the three genotypes were dead under 2 weeks of exposure to submergence stress. When the duration of stress was increased further to 18 days, difference in tolerance ability of FR13A, AC42088 and AC 42087 were

observed. After 18days of stress, AC42088 was still having 100% survival, while it was dropped to 83% in AC42087 and 66% in both FR13A and AC1303.

Water is an essential component of plant growth. Although, rice plants require substantial amount of water during its growth, but excess water in the form of partial (water logging stress) or complete submergence is reported to hamper plant growth and survival (Setter and Waters 2003; Colmer and Pedersen 2008; Sarkar et al. 2011). Many rice cultivars, despite having fairly good aeration trait in the form of formation of aerenchyma, succumb to complete submergence after a few days. Rapid stem elongation to come out of water, can exhaust the energy and utilize the available carbohydrate reserve leading to plant death if depth of water and/or duration of flooding is more (Jackson and Ram 2003; Bailey-Serres et al. 2010). But, the huge genetic diversity of rice gifted us with some flash-flood tolerant Eastern Indian genotypes like FR13A, Kalaputia having the ability to withstand a period of complete submergence as long as two weeks. These genotypes can utilize their carbohydrate reserve more efficiently by minimizing the metabolic cost of growth during the period of submergence and can restart their normal growth, when flood water recedes (Singh et al. 2001; Fukao et al. 2006). Recently, we have identified some more rice germplasm from lowland rice growing ecology of Eastern India, which are superior than FR13A, the model flood tolerant rice genotypes used globally as donor parent and also for discovery of *SUB1*QTL in rice (Bailey-Serres et al. 2010). Our evaluation showed, the genotypes like AC42088, AC42087 and AC1303 have significantly higher submergence tolerance ability than FR13A and can withstand complete submergence even up to three weeks. Understanding the mechanism of tolerance in these genotypes can significantly improve flood-tolerant rice variety development programme in rice.

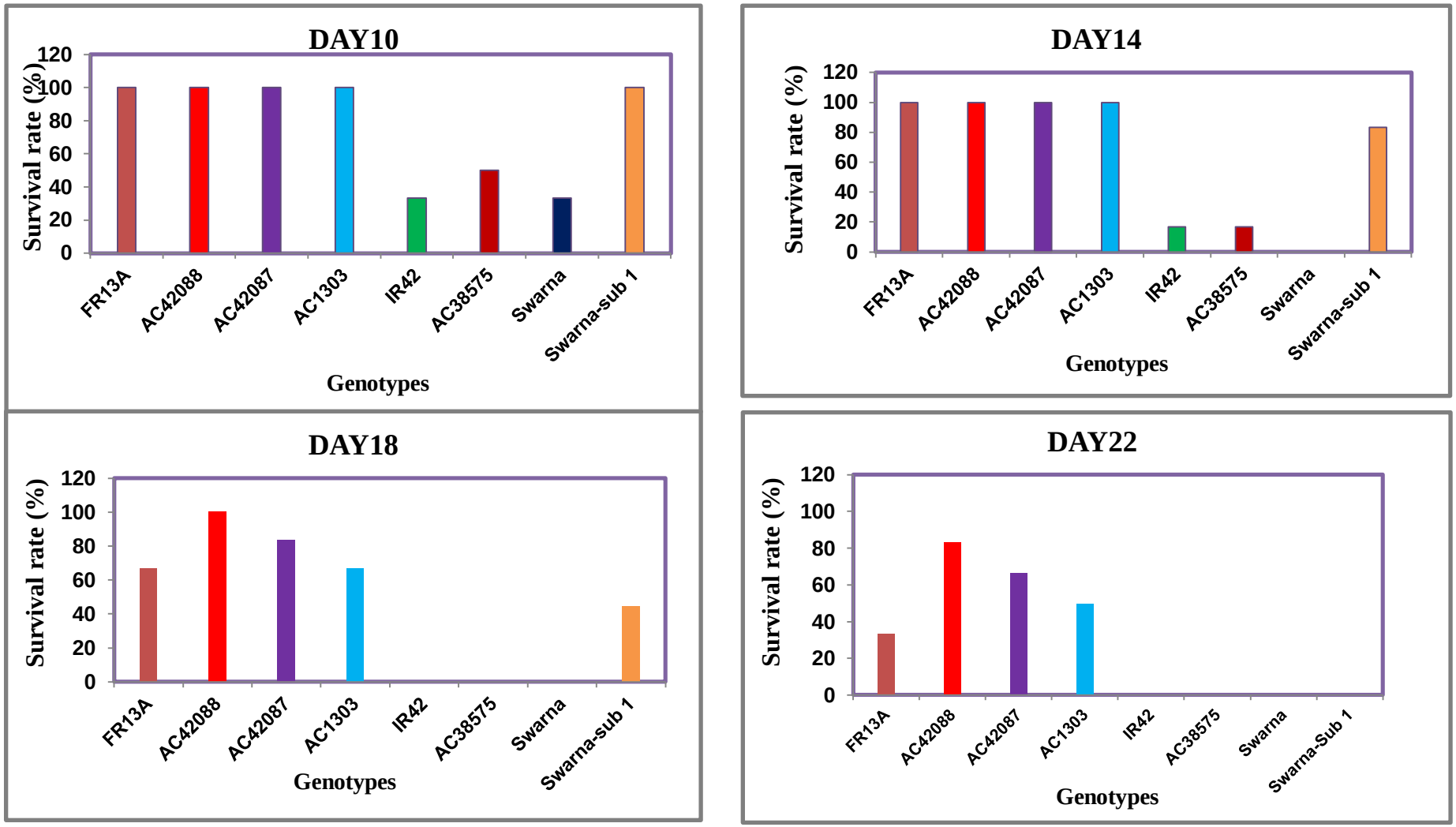


Fig 4.1: Effect of submergence on Survival rate (%) in eight rice genotypes.

4.1.2: Elongation ability:

Significant differences were found in elongation ability (EA) of different genotypesexposed to different durations of submergence stress (Fig.4.3). Susceptible genotypes like IR42 and Swarna showed highest EA of 55% and 34%, respectively at 10^{th} day of submergence. Subsequently, the EA was found to be 65% and 43%, respectively in these genotypes after 14 days of submergence. Among the studied genotypes Swarna-Sub 1 and AC1303 showed least elongation ability during the entire duration of submergence stress. It was <10% in both the genotypes up to 14 days of stress. It was increased slightly i.e. 12 and 15% respectively in Swarna-Sub 1 and AC1303 after 18 days of stress and finally it was ~20% in AC1303 after 3 weeks of stress, while Swarna-Sub 1 died completely at that time. It is interesting to note here that the genotypes possessing beyond two weeks submergence tolerance had invariably lesser elongation ability than our tolerant check FR13A at any point during the entire period of submergence stress.

Sarkar et al. (2011) reported minimal increase in the plant height in rice cultivars having *SUB1*QTL present. These genotypesshowed lesser elongation ability as compared to other genotypes.(Singh et al. 2011) found that there was a negative correlation between survival percentage and rapid elongation of stem under submerged condition in rice genotypes. From the previous studies, it was understood that submergence tolerant genotypesshow limited elongation during submergence and likely to use only a small quantity of available carbohydrate for elongation, leaving rest for survival during submergence (Setter *et al.*,1997; Sarkar*et al.*, 1996; Setter *et al*., 1996).

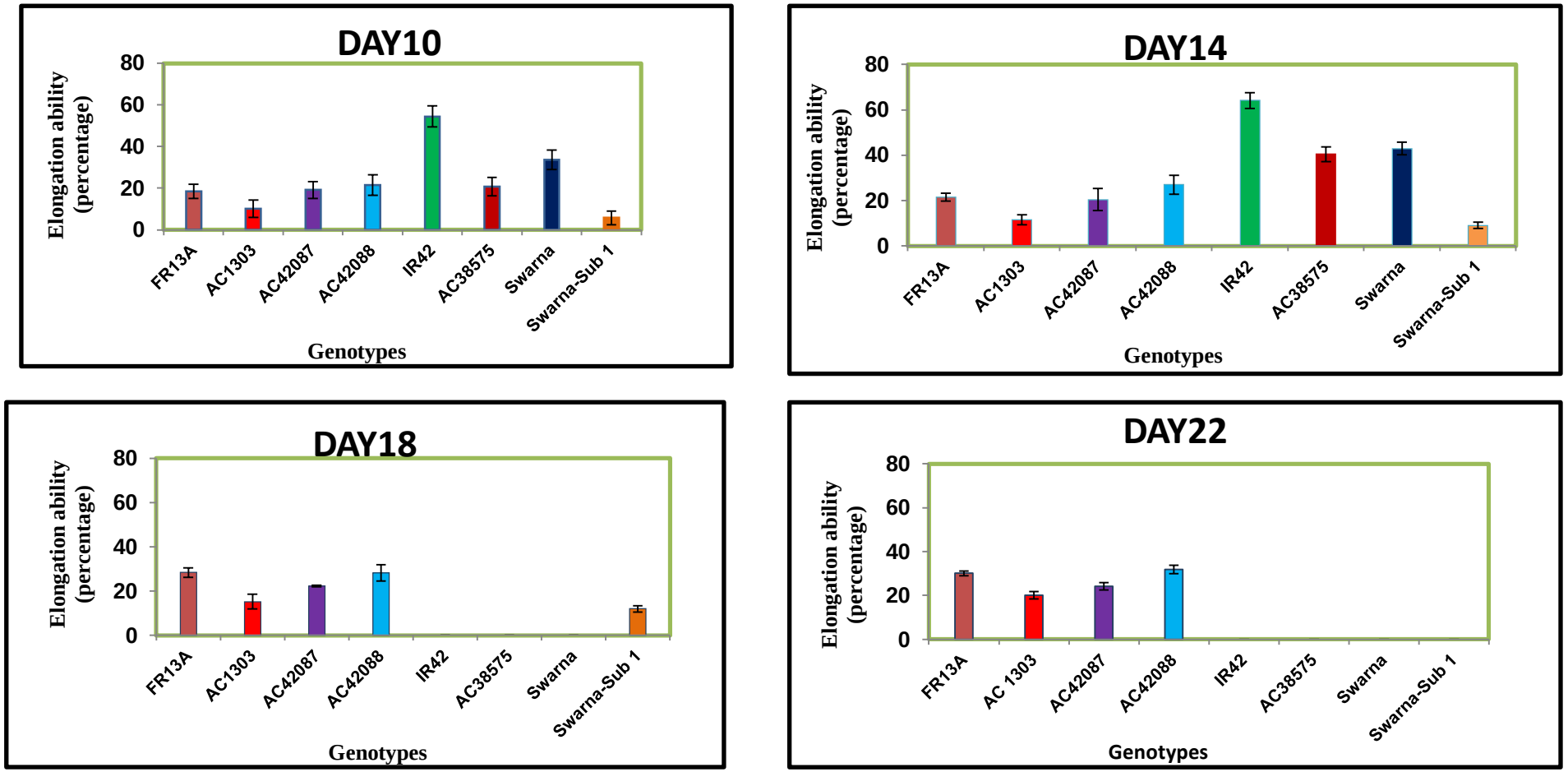


Fig 4.3: Effect of submergence on elongation ability in eight rice genotypes

Vertical bars represent SEM (Standard error mean)

4.1.3: Plant height:

Data pertaining to plant height before submergence and after 10^{th},14^{th},18^{th} and 22^{nd} days of submergence are presented in table 4.1. The results showed that the genotype AC42087 had the highest initial plant height i.e. 41 cm followed by FR13A (39.07 cm), whereas Swarnahad least plant height (21 cm) before submergence. The increase in plant height were comparatively more in susceptible genotypes as compared to tolerant ones after submergence.After 10^{th} day of submergence the susceptible genotype IR42, achieved a height 54 cm which was only 31 cm on day zero.On 14^{th} day maximum plant height was observed in AC38575. All the susceptible genotypes (AC38575,IR42 andSwarna) died after 14 day of submergence.

Table 4.1: Changes in plant height in different rice genotypes due to submergence.

Genotype	DAY 0	DAY 10	DAY 14	DAY 18	DAY 22
FR13A	39.07	48.65	50.17	53.81	54.66
AC1303	38.33	44.95	45.77	47.66	49.06
AC42087	41	51.33	52.08	53.08	54.18
AC42088	36.33	46.66	49.33	49.82	51.62
IR42	31.33	54	58	*	*
AC38575	40.33	51.33	61	*	*
Swarna	21	28.66	31	*	*
Swarna-Sub 1	22.66	23.66	24.66	26.29	*
$LSD_{0.05}$(G x T)					**2.95**

Note: All values are mean of three replications.

*All the plants died in that particular period of time

The genotype AC1303 had the least plant height after submergence at the end of

entire duration of submergence stress, thereby showing least internode elongation after three weeks of stress, which might be one of the reasons for its greater survival under prolonged submergence. Rapid increase in plant height attributes to an escape strategy called low-oxygen escape syndrome,where there is a faster elongation of internode so that the plant can come out of water within initial 3-4 days of complete submergence (Bailey-Serres and Voesenek 2009). It was reported that faster utilization of the available carbohydrate reserve for rapid underwater internode elongation can exhaust the plant quicker by depleting the energy source leading to increased plant mortality in these type of genotypes (Singh *etal*. 2001).

4.2: Physiological Measurements:

4.2.1: Leaf gas film thickness:

Enough diversity was observed in the leaf gas film of studied genotype on both the adaxial and abaxial surface of leaf of the studied genotype on control condition. Upon imposition of submergence stress, different genotype showed differential rate of depletion of leaf gas film. Mean values of leaf gas film thickness (µm) on adaxial and abaxial surfaces are represented in the Fig. 4.4(a).Among the eight rice genotypes, AC1303 was found to possess highest leaf gas film thickness both on adaxial and abaxial sides (29.39 µm) before submergence, followed by AC42088(29.19 µm) and least gas film thickness was observed in AC38575(9.46 µm). The underwater retention of leaf gas film was least in Swarna, AC38575, IR42 and the gas film was almost completely depleted within 4 days of submergence. A significantly greater leaf gas film retention ability was observed in the genotypes like AC1303 followed by AC42088,AC42087, which could able to retain their gas film even up to 8days after submergence.The known submergence tolerant genotypes like FR13A and Swarna Sub-1 were also found to have fairly good leaf gas film retention ability, in which the gas film was completely depleted after 7 days of submergence stress.Leaf gas films contribute to submergence tolerance in plants as it increases the resistance of leaf and water interface thereby lowering for inward diffusion of CO_2 and O_2 into the leaves of a submerged plant(Verboven etal., 2014). This can substantially aidunderwater photosynthesis and internal aeration at least for initial few days during complete submergence (Pedersen etal., 2009; Winkel etal., 2013, 2016). Under submerged

condition, the stomata of the rice leaf close as soon as it comes in contact of water leading to complete seizure of gas exchange and photosynthesis. Presence of such gas film on the leaf surface enables the plants to keep their stomata open even after a few days of submergence (Colmer and Pedersen, 2008). Here we found the genotypes having an ability to retain their leaf gas film for longer duration could able to survive longer duration of stress.

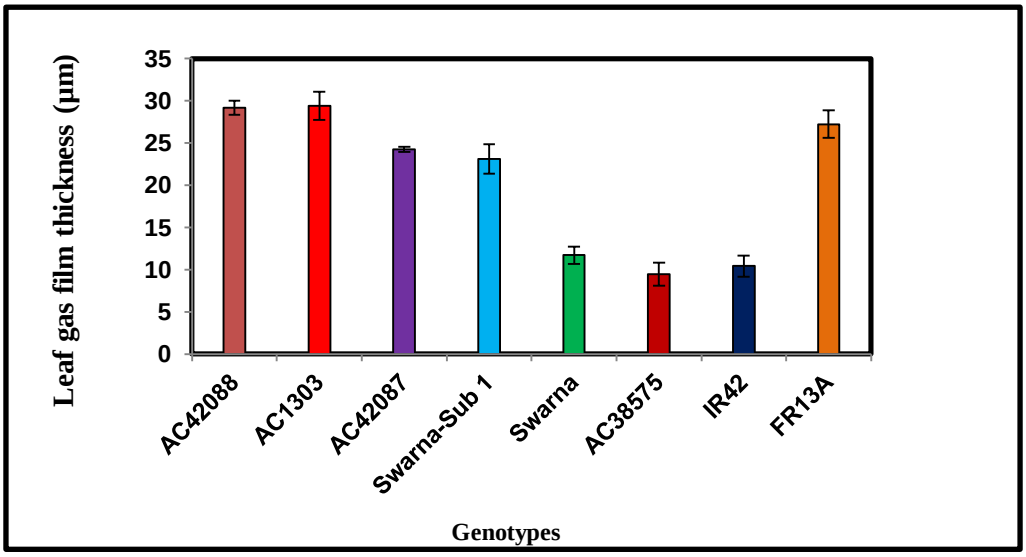
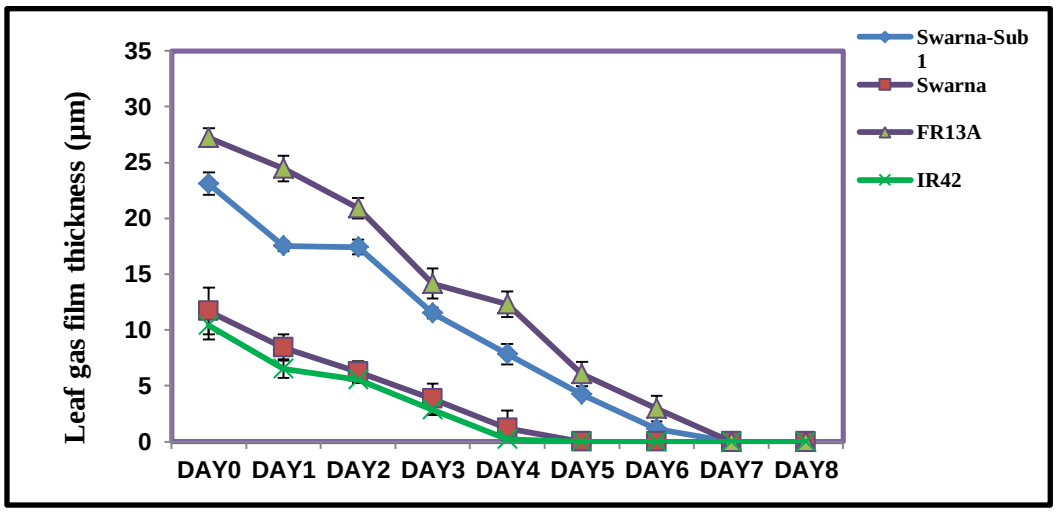


Fig 4.4(a): Diversity in leaf gas film thickness on adaxial and abaxial surfaces of eight rice genotypes.



4.4(b): Reduction of the leaf gas film thickness on adaxial and abaxial surfaces of eight rice genotypes

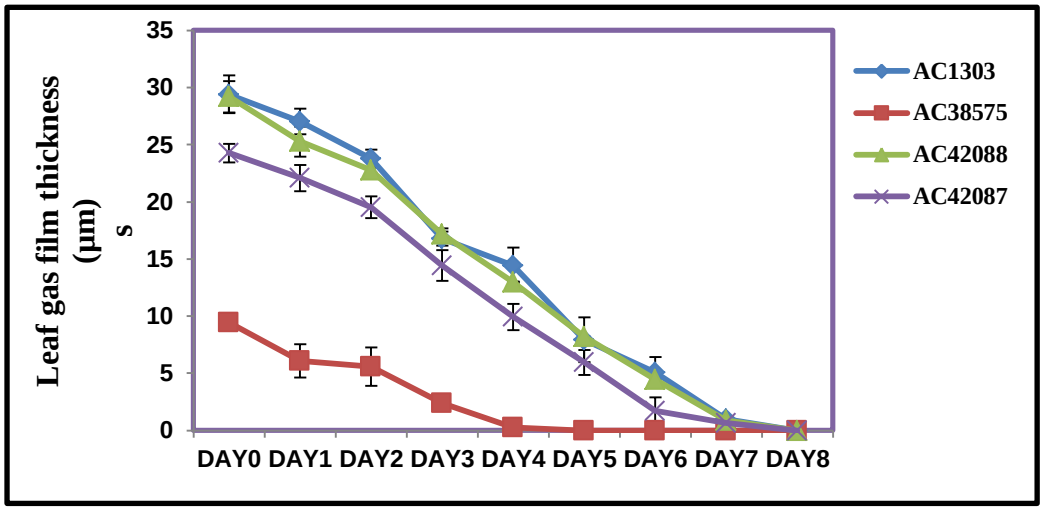


Fig 4.4(c): Reduction of the leaf gas film thickness on adaxial and abaxial surfaces of eight rice genotypes

4.2.2:Leaf porosity:

Similar to the leaf gas film, porosity of the mesophyll tissue also plays a significant role in submergence tolerance of rice. The result showed considerable differences in porosity of mesophylltissue among eight genotypes (Fig. 4.5a,b& c).The genotype FR13A was found to have highest leaf porosity percentage (50.95 %) followed by AC1303(49.09 %) and AC42088(47.83 %) under control condition.Whereas,the genotype AC38575 was found to be possess least leaf tissue porosity (41.89 %) under control conditions. The data recorded under stressed condition revealed that, FR13A showed highest percentage of tissue porosity (25.56), followed by AC1303 (23.37%), AC42087 (18.58%), AC42087 (17.34%) even after 7 days of complete submergence.The genotypes AC38575, which was having least tissue porosity under control condition also found to possesslowest tissue porosity (10.56%)7 days after complete submergence. Decrease in tissue porosity under submergence stress was varied between different genotypes. Most of them showed significant reduction in porosity percentage after 5 days of stress imposition. Among the genotypes both FR13A and AC1303 showed least reduction in tissue porosity during our course of study. Greater tissue porosity leads to increased ethylene mediated aerenchymaformation in rice (Colmer&Voesenek2009; Yamauchi *et al*. 2018). Previous studies also showed that submergence tolerant rice genotypes like FR13A, possessed much higher tissue porosity as compared to submergence

intolerant lines such as IR42 or Swarna (Winkel et al. 2014). In the present study, although we found significantly higher tissue porosity in FR13A, AC1303, AC42087 and AC42088 as compared to the rest of the genotypes, but surprisingly underwater retention of leaf tissue porosity didn't correlate*in to* with final survival ability of these genotypes, especially after two weeks.

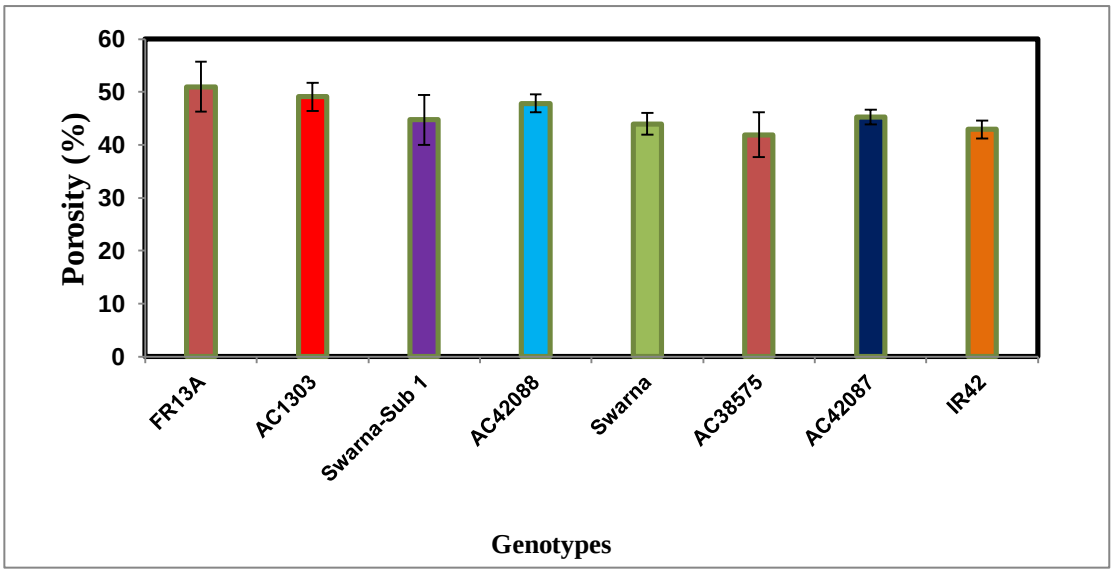


Fig 4.5(a): Diversity in leaf tissue porosity of eight rice genotypes

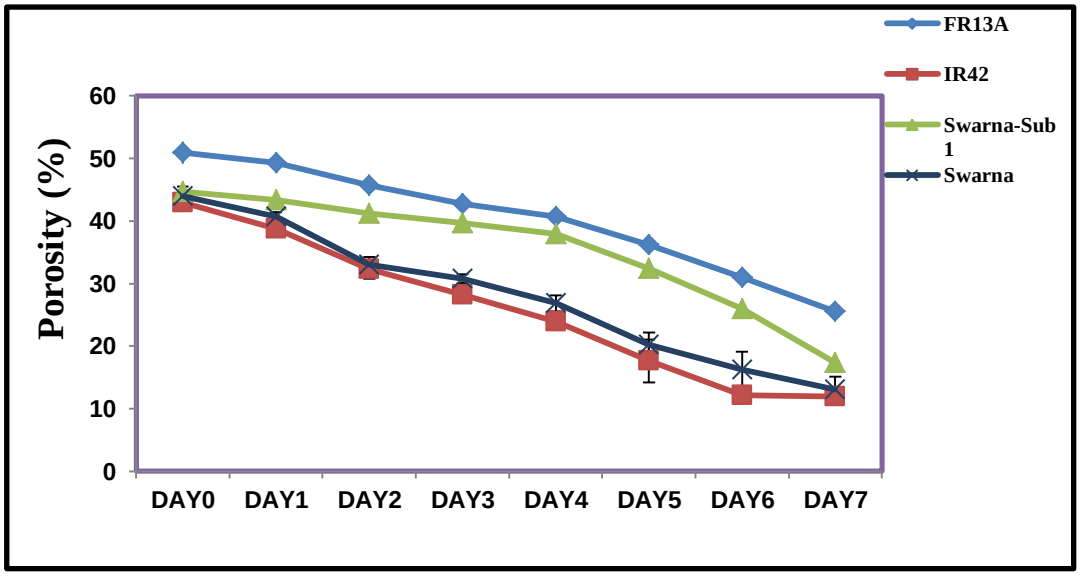


Fig 4.5 (b): Reduction of the leaf tissue porosity of eight rice genotypes

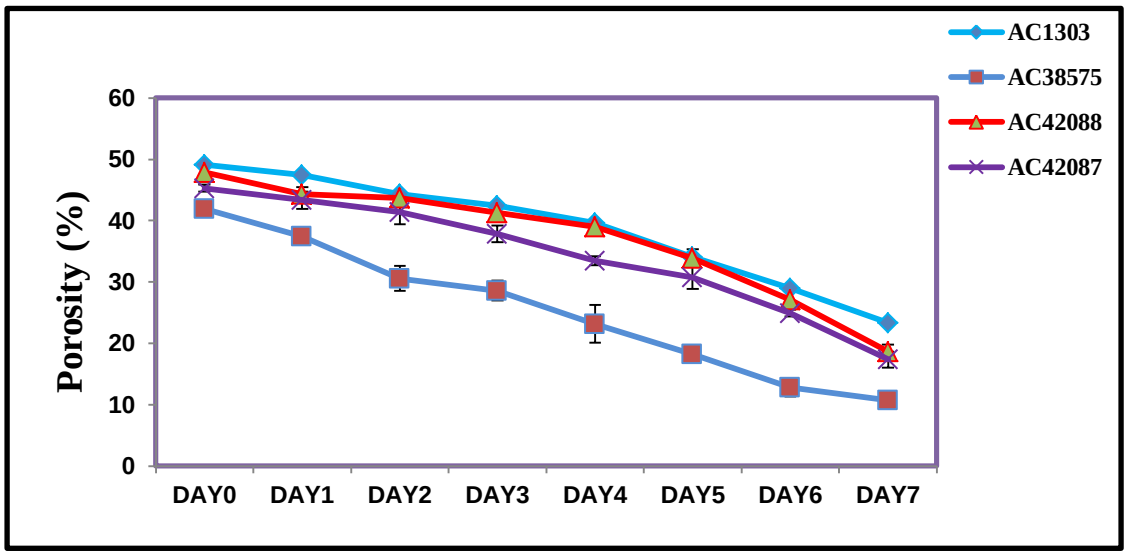


Fig 4.5 (c): Reduction of the leaf tissue porosity of eight rice genotypes.

4.2.3: Leaf Hydrophobicity

Hydrophobicity of the leaf surface was measured in terms of contact angles of water droplet on the leaf surface (Fig 4.6) and the data was tabulated in Table 4.2. The leaf blade surface was found to behydrophobic initially on all genotypes as the contact angle was found to be >125° in AC1303, AC42088 and AC42087. It was significantly lower in susceptible genotypes like IR42 (112°) and Swarna(116°). Interestingly, we did not observe much difference in initial contact angle in Swarna (116°) and Swarna-Sub 1 (117°), they had distinctly different submergence tolerance ability. Among the studied genotypes, AC1303showed maximum contact angle(128.33°),followed by AC42088(126°) andAC42087(125°) undernormal condition. But, we found a rapid decline in leaf hydrophobicity upon complete immersion of leaves under water.Reduction in contact angle was not significant after 4 hour of immersion in any genotype, but it reduced significantly since 24 h of submergence. Within 48 hr of submergence, the leaf surface of the genotypes like Swarna, IR42 and AC38575had become hydrophilic as its contact angles dropped well below 75°.But for rest of the genotypes, theleaves remained hydrophobic at this stage.After 96 h of submergence stress, two genotypes were able to maintain the contact angle of 100° or more. AC1303 showed highest leaf contact angle of 105°, followed by AC42088 had a contact angle of 98° after 96 h

of submergence, whereas it was dropped to 81° in FR13A and 89° in Swarna-Sub 1 and became 0 in Swarna, IR42 and AC38575.

Hydrophobic surfaces can retain a thin layer of gas when submerged under water. It can promote a mechanism of enhance gas exchange with water, evident in some plants and aquatic invertebrates (Thorpe & Crisp, 1947; Hebets& Chapman, 2000; Pedersen &Colmer, 2012).The importance of leaf hydrophobicity and leaf gas film for supporting underwater photosynthesis and submergence tolerance was already established in rice (Pedersen etal., 2009; Winkel et al., 2013; Colmer et al., 2014). In the present study, we found a good correlation between final survival ability of the studied genotypes (three weeks after submergence) with leaf hydrophobicity after 96 h of immersion underwater. The genotypes like AC1303 and AC42088, which showed maximum survival after 3 weeks of submergence stress were also very efficient in retaining their leaf hydrophobicity for significantly longer duration.

Table 4.2:Changes in contact angles in different rice genotypes due to submergence.

Genotypes	CONTACT ANGLE				
FR13A	122.33	119.66	117.33	112.66	81.00
Swarna	116.66	112.00	111.33	50.00	0.00
Swarna-Sub 1	117.00	114.66	107.33	106.50	89.33
IR42	112.00	109.66	106.66	60.00	0.00
AC42088	126.00	123.00	122.33	118.33	98.33
AC42087	125.00	119.33	115.00	112.33	86.00
AC1303	128.33	121.33	119.66	107.33	104.67
AC38575	116.00	107.33	99.66	64.66	0.00
$LSD_{0.05}$(GxT)					**8.34**

4.2.4:Epicuticular wax content:

Total epicuticular wax content of the leaves of submerged plants were estimated in all the genotypes on a daily basis and the data was tabulated in Table 4.3, which showed the differential response of the genotypes regarding the diversity in total wax content. Under controlled conditions, the genotype AC1303 showed highest amount of wax content (21.61µgm), followed by AC42088 (17.10µgm), AC42087 (16.59µgm), which is comparatively more than that of the

tolerant check varieties, like: FR13A (13.12µgm) and Swarna-Sub 1(12.7µgm) and the least amount was noticed in AC38575 (8.59µgm).Significant rate of reduction was observed in the genotypes after 4 days of complete submergence and the rate of degradation was comparatively slower in genotypes like: AC1303, AC42087, AC42088 as compared to the submergence tolerant checks. The variety AC1303 performed well even after 7 days of complete submergence showing 6.69µgm of wax content, which might help this genotype to survive prolonged submergence stress of beyond two weeks.Kurokawa et al. (2018) reported that epicuticular wax content is directly responsible for greater leaf hydrophobicity in rice. The content of epicuticular wax was found to govern by the *LGF1* gene action, which is involved in the biosynthesis pathway of a C30 primary alcohol responsible for plant wax formation. They have also demonstrated that expression of *LGF1* was directly correlated with leaf gas film thickness in rice.

Table 4.3: Mean values of wax content (µ g cm^{-2}) of eight rice genotypes on different time period

	WAX							
Genotype	**DAY0**	**DAY1**	**DAY2**	**DAY3**	**DAY4**	**DAY5**	**DAY6**	**DAY7**
FR13A	13.12	12.19	9.91	7.95	7.16	5.51	4.27	3.85
AC42087	16.59	15.63	13.63	11.16	9.33	7.22	6.68	5.52
AC42088	17.10	16.27	14.52	12.63	10.86	9.91	7.23	6.03
AC38575	8.59	6.44	4.75	4.19	2.21	0.81	3.28	3.28
AC1303	21.61	20.19	18.25	15.42	12.54	10.37	7.71	6.69
IR42	9.18	6.81	5.89	3.90	2.82	0.85	2.66	1.66
Swarna	11	9.12	7.06	4.85	2.88	0.93	1.24	1.90
Swarna-Sub 1	12.7	10.80	8.92	6.41	4.72	4.23	2.40	2.06
$LSD_{0.05}$ (G x T)								**2.06**

4.2.5: Estimation of total chlorophyll content:

A significant reduction in chlorophyll content was observed under submergence stress as compared to control conditions with notable differences among thegenotypes.The observed total chlorophyll content was presented in the

Table 4.4. In particular, susceptible genotypes (IR42,Swarna and AC38575) exhibited rapid decline in total chlorophyll content under stresses condition as compared to tolerant check (Swarna-Sub 1 and FR13A) varieties. On day 10, FR13A shows 20.54% reduction in chlorophyll content, whereas in Swarnathe reduction was 54%, followed by IR42 (38.85%).The genotypes showing better survival rate than FR13A or Swarna-Sub 1 under longer duration of submergence stress showed even lower rate of chlorophyll reduction under submergence stress. AC42088 showedthe least rate of degradation (16.06%), followed byAC42087 (15.64%) and AC1303 (22.61%) among the studied genotypes. Chlorophyll degradation is the direct effect of the submergence stress as observed in rice in previous reports (Sarkar et al. 2001). Complete submergence results in an increase of ethylene synthesis which induces the expression and activity of chlorophyllase, an enzyme responsible for degradation of chlorophyll (Das *et al*.,2005; Sarkar et al.,2001; Ella *et al*., 2003).

Table 4.4:Changes in total chlorophyll content (mg g^{-1} fresh weight) in leaves of different genotypes of rice in response to submergence stress.

Genotypes	DAY 0	DAY 4	DAY 8	DAY 12	DAY 16	DAY 20
FR13A	2.31	2.13	1.83	1.29	0.67	0.32
IR42	2.06	1.58	1.26	0.79	*	*
AC1303	2.23	2.00	1.72	1.30	0.89	0.56
AC42088	2.35	2.24	1.97	1.50	0.97	0.67
AC38575	2.01	1.77	1.33	0.74	*	*
AC42087	2.08	1.88	1.75	1.32	0.85	0.49
Swarna	2.46	1.42	1.13	0.85	*	*
Swarna-Sub 1	2.01	1.78	1.64	1.16	0.44	*
$LSD_{0.05}$ (G x T						**0.077**

*All the plants died in that particular period of time.

4.3: Biochemical analysis:

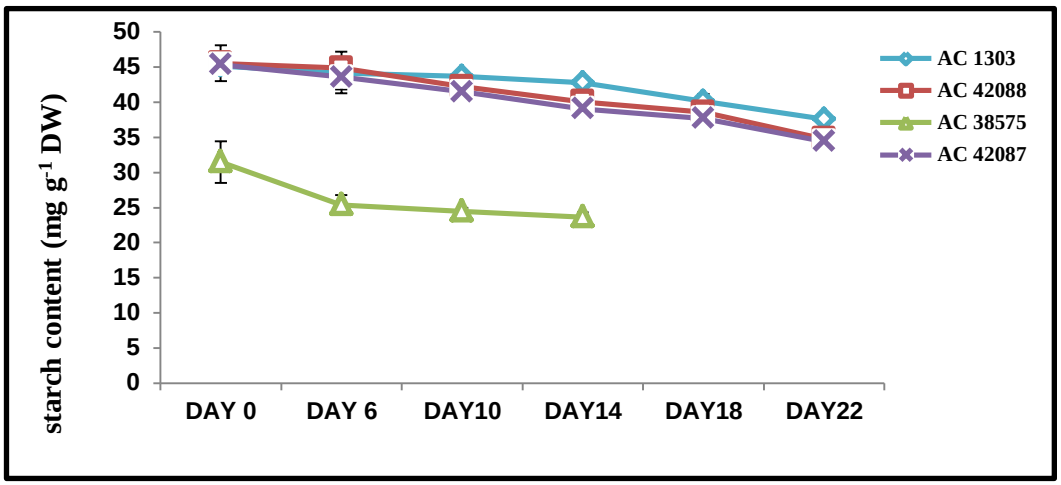
4.3.1: Starch content:

There was very significant and distinct difference among the initial starch content of the leaves observed among the studied genotypes under control

condition. Starch content of eight different rice genotypes estimated at every 4 days interval after imposition of submergence stress is presented in (Fig. 4.7).Variations in the starch content of rice genotypes were studied and the results were compared with the tolerant and susceptible check varieties under stress conditions. A gradual reduction in starch content was observed in all the genotypes under submergence stress compared to the initial starch content just before imposition of stress. The known tolerant genotypes likeFR13A and Swarna-Sub1showed low rate of reduction under submergence stresses during the initial period of stress ascompared to the susceptible genotypes like IR42 and Swarna. Similarly, the genotype AC1303 had only 3.34% per day reduction in starch content followed by AC42088(7.34%)and AC42087(8.54%), which showed even lower rate of reduction that FR13A and Swarna-Sub1.Genotypes like Swarna,IR42 and AC38575 could not withstand entire duration of stress and completely after 14 days of submergence.At the end of three weeks of submergence, the genotype AC1303 was found to have highest amount of leaf starch content of 37.56 mg g^{-1} DW, which was significantly higher than internationally known submergence tolerant line FR13A (33.12 mg g^{-1} DW).

Both the initial level of non-structural carbohydrate content in leaf and well its gradual decrease under complete submergence plays key role in submergence tolerance in rice (Sarkar et al. 2011). Conserving greater amount of carbohydrate byslower depletion is positively correlated with the level of submergence tolerance (Santosaet al.,2007; Ram et al., 2002; Mallik*et al.*,1995). During submergence slower utilization of carbohydrate was reported to occur in tolerant genotypes which could sustain and supply the metabolic energy required for survival of plants under water (Das et al.2005,Winkel et al.2014). An evaluation of submergence-tolerant and submergence-intolerant rice grown under unstressed conditions revealed that the seedlings of tolerant landraces normally had 30–50 % more NSC compared with sensitive genotypes (Sarkaret al., 2005).In the present study, we found a positive correlation between carbohydrate reserve and submergence tolerance ability of studied rice genotypes. The genotypes like AC42088, AC1303 and AC42087, which were found to be superior in terms of their prolonged submergence ability as compared to FR13A or Swarna-Sub1, were also having

greater carbohydrate reserve in their leaves at the end of three weeks of stress.This might help these genotypes in faster recovery upon de-submergence.



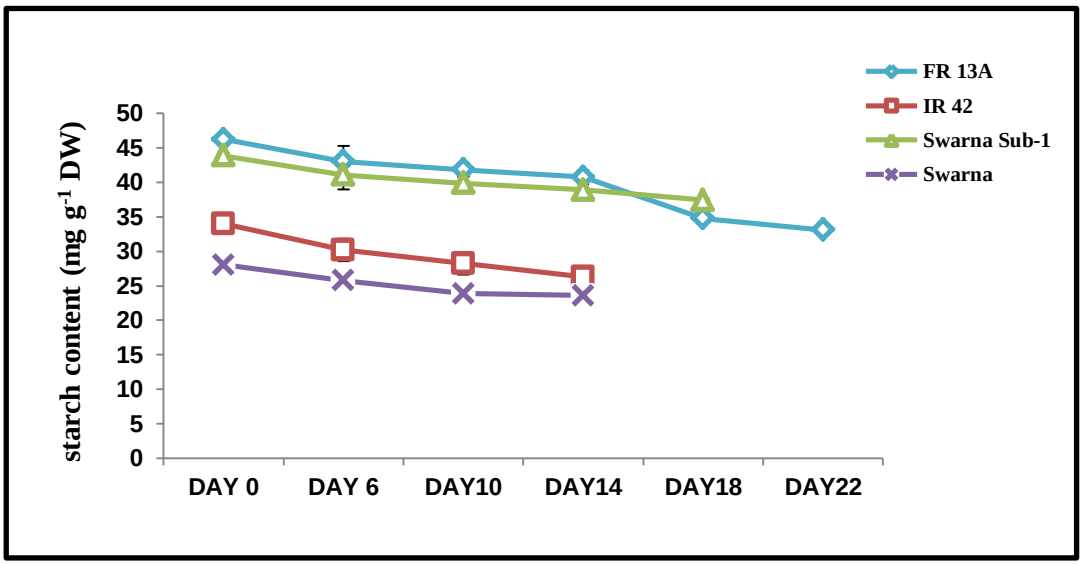


Fig. 4.7: Mean values of starch content (mg g^{-1} dry weight) of eight rice genotypes on different time period

Note: Vertical bars represent SEM (Standard error mean)

4.4: Molecular Study

4.4.1:SSR Primers profiling:

Swarna-Sub1 genotype is a Near Isogenic Line (NIL) of Swarna. It is similar to Swarna for almost all regions of the genome except the region carrying *Sub-1* QTL. Hence, the markers selected for the present study,werefrom the genomic regions lying in proximity of *Sub-1*, which should be able to differentiate these two genotypes. We used SSR markers from regions near to *Sub-1* QTL and characterized the two genotypes besides five other rice accessions and one susceptible cultivar (IR42). Out of the 26 SSR markers screened, five markers namely RM219, RM316, RM8300, RM23958 and ART5 were found to be polymorphic in 3.5% Agarose gel. All the polymorphic markers were distinguishing Swarnaand Swarna-Sub1. The dendogram constructed using UPGMA and Jaaccard's similarity coefficient showed that the genotype Swarna lies as outlier with respect to all the other genotypes including Swarna Sub-1. The tolerant genotypes formed different sub-clusters and AC1303 was distinct from the rest. The genotype IR42 was lying in same cluster with the resistant genotypes. The size of allele for a particular SSR marker can't be traced for identical/differential feature of descent or type. Hence directly associating a band size of SSR marker to tolerance or susceptibility is not appropriate. However, the ability of the markers to clearly distinguish Swarna Sub-1 and Swarna confirms their linkage/association with *Sub-1* QTL. Additionally, sufficient diversity among the genotypes even with such limited set of markers from a specific region of the genome was detected. This indicates that the genotypes used in our study are diverse, albeit presence of same submergence tolerance gene in all the tolerant genotypes.

Table 4.5: List of SSR markers showing polymorphism among the genotypes

SSR Markers	Forward Primer	Reverse Primer
RM219	CGTCGGATGATGTAAAGCCT	CATATCGGCATTCGCCTG
RM8300	GCTAGTGCAGGGTTGACACA	CTCTGGCCGTTTCATGGTAT
RM 316	CTAGTTGGGCATACGATGGC	ACGCTTATATGTTACGTCAAC
RM2395	CTACCACTGTTTCATTGTGTCT	GAATTGAAGGAGAAGCAGGAA
8	CG	GC
ART5	CAGGGAAAGAGATGGTGGA	TTGGCCCTAGGTTGTTTCAG

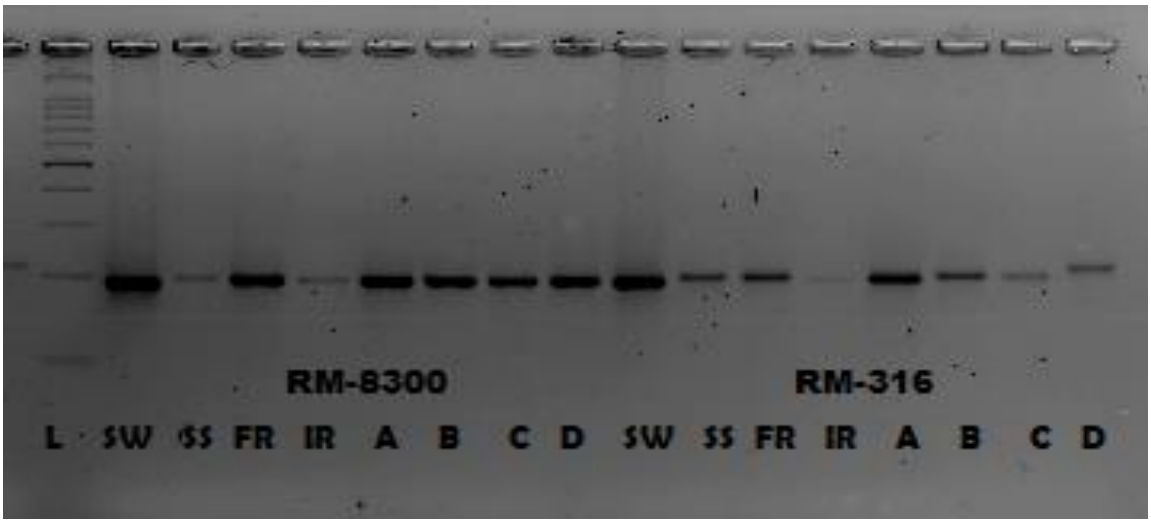


Fig. 4.8(a): Agarose gel showing polymorphismwith primer RM-8300 & RM-316

{L-Ladder(100bp), SW-Swarna, SS-Swarna Sub1, FR-FR13A, IR-IR42, A- AC 42087, B-AC 42088, C-AC 38575, D-AC 1303}

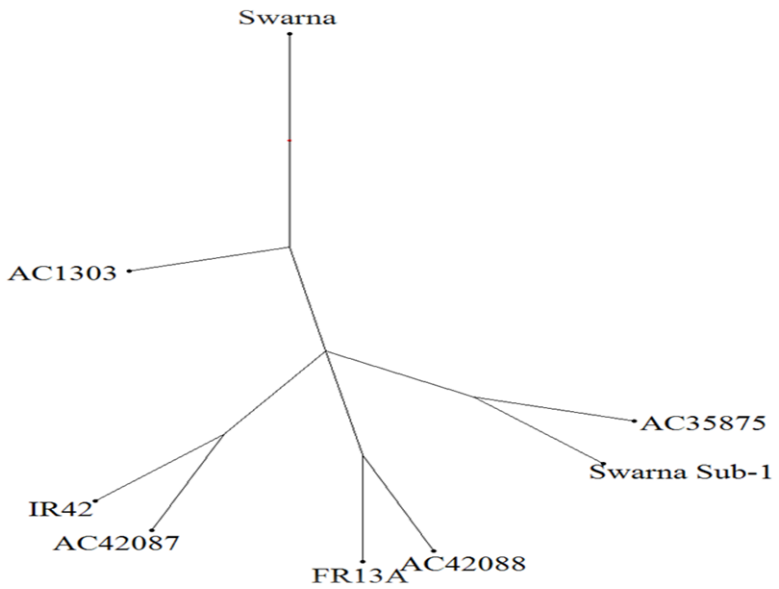


Fig. 4.8(b): Dendrogram depicting genetic relatedness among genotypes based on Sub-1 linked marker

4.4.2:Gene expression studies

4.4.2.1:q-PCR analysis

Out of the eight genotypes taken for the present study, we have selected three *viz*. FR13A, AC1303 and IR42 for gene expression study.The genotypes were selected in order to compare the response of SUB1 genes in the genotypes showing three weeks of complete submergence with respect to the universal submergence tolerant check FR13A and susceptible check IR42. Gene expression profile of different submergence related genes (*SUB1A, SUB1B* and *SUB1C*) present in *SUB1* QTL region of the genome showed several fold up-regulation in the leaves of rice genotypes three days after imposition of submergence stress. The results showed that massive induction of expression of *SUB1A* gene in FR13A, which was in the tune of ~2700-fold (as compared to control) within three days of imposition of stress {Fig.4.1.5.1(a)}. On the contrary, the upregulation was only ~135-fold in susceptible genotype IR42. Surprisingly, the expression of SUB1A gene was intermediate in AC1303, a genotype having better prolonged submergence tolerance ability than FR13A.

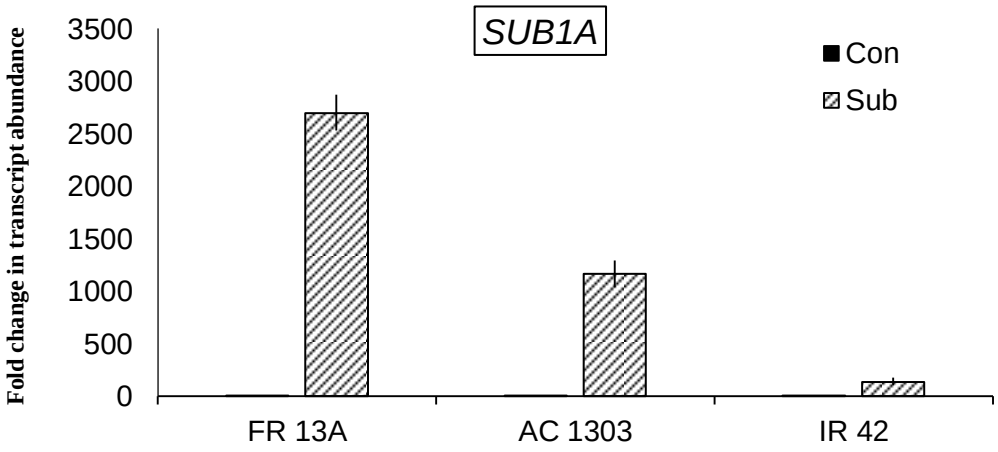


Fig. 4.9(a): Expression of *SUB 1A*gene in response to submergence stress

The expression of *SUB1B*, also showed similar pattern of induction, but with a bit lesser magnitude {Fig4.1.5.1(b)}. The level of upregulation was ~120-, 80- and 55-fold in FR13A AC1303 and IR42, respectively. But, unlike *SUB1A* and *SUB1B*, relatively greater expression of *SUB1C* gene was observed in most susceptible genotype IR42 {Fig 4.1.5.1(c)}.There were almost 40-fold

upregulation in *SUB1C* expression observed in IR42, while it was little less in FR13A (35-fold) and AC1303 (32-fold). Earlier studies reported that higher expression of *SUB1A* gene is directed related to the submergence tolerance ability of the rice genotypes (Fukao et al., 2006). It was also proposed that *SUB1A,* represses rapid internode elongation under complete submergence by reduced ethylene-mediated GA biosynthesis and *Sub1C* suppression and thereby reduces faster carbohydrate depletion (Perata and Voesenek, 2007). Xu et al. (2006) reported that rather limited expression of Sub1C was associated with tolerance. The results obtained from the gene expression profile of *SUB1A*in the present study, contradict the clear positive correlation theory of *SUB1A* expression and submergence tolerance ability. Here, we found though the submergence tolerance ability of AC1303 was superior as compared FR13A, especially when the stress was beyond two weeks, but interestingly the expression of SUB1A was more than double in FR13A (~2700-fold) as compared to AC1303 (~1200-fold). This suggests there might be some *SUB1A* independent factors contributing to prolonged submergence tolerance ability in these genotypes. A comparatively dissection of different submergence tolerance strategy may give an insight of the most important components of tolerance mechanism in rice.

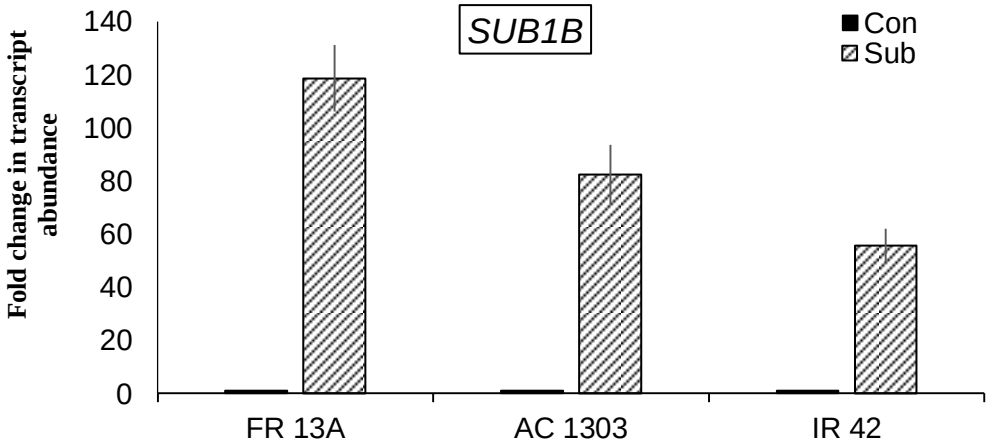


Fig. 4.9(b): Expression of *SUB 1B and SUB 1C* genes in response to submergence stress.

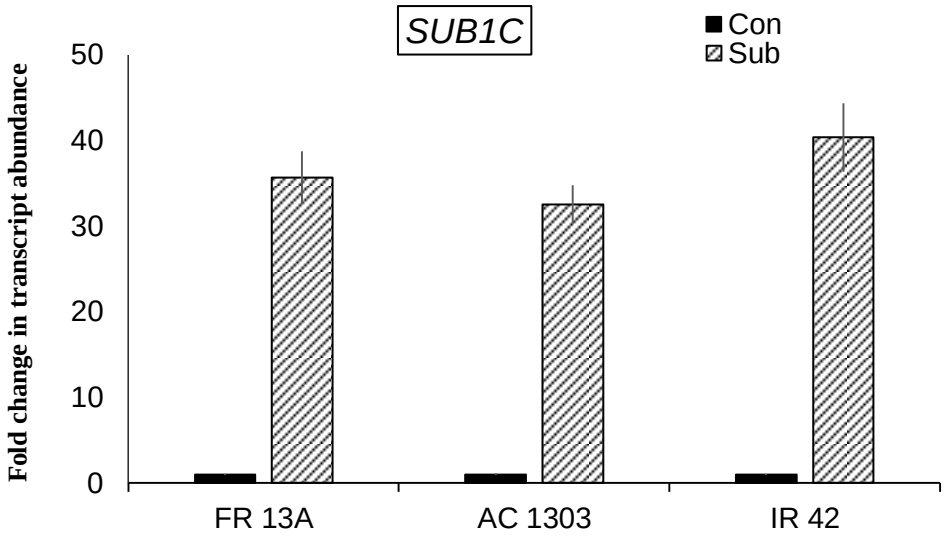


Fig. 4.9(c): Expression of *SUB 1B and SUB 1C* genes in response to submergence stress

CHAPTER – V
SUMMARY AND CONCLUSION

5.1. SUMMARY

Rice, being one of the most important cereals, is cultivated as the staple food globally under diversified soil and climatic condition. Rice plant is affected by different kinds of environmental challenges including both biotic and abiotic stresses. Excess of water in the form of submergence in rice fallows sets a bottleneck for both survival and productivity of indigenous land races. Complete submergence due to flooding is most prevalent in the low-lying rice growing areas of the world. Crop loss due to excess water and water logging is also considerably high in such regions. Submergence is a type of flooding stress in which part or the entire plant remains under water. Visible symptoms of injury caused by submergence include an initial phase of faster elongation by one or more leaves accompanied by yellowing of older leaves and slow or negative growth in dry mass of roots and shoots. Currently we are having submergence tolerant rice cultivars which can withstand a period of 10-14 days of complete submergence and can resume its normal growth when flood water recedes. But with ever increasing threats of different climatic adversities, the frequency of incidence of such extreme events increased considerably. Nowadays, the event of complete submergence in the rice fields for a prolonged period often spanning up to three weeks were reported. Fortunately, in the nature we are blessed with wide diversity of rice germplasms. Preliminary work showed that submergence tolerance beyond two weeks can be achievable in rice. A few genotypes were reported to possess prolonged duration of submergence stress (about 3 weeks). As these genotypes can withstand even longer duration of stress (~3 weeks), where FR13A fails to survive, so it creates enough doubt that whether they follow a similar mechanism of FR13A or not. Hence, the present study is hypothesized to investigate the physiological strategies as well as the molecular factor(s) associated with prolonged

(~3 weeks) submergence tolerance in the unique genotypes like AC42088, AC42087 and AC1303, which are having superior submergence tolerance ability beyond 2 weeks.

For this, eight genotypes of rice (*Oryza sativa* L.) comprising of come cultivars and unique germplasms viz. FR13A, Swarna, Swarna Sub-1, IR-42, AC1303, AC42088, AC42087 and AC38575 were characterized for different components of submergence tolerance mechanism under controlled condition. Different physiological, biochemical and molecular parameters were investigated to identify the physiological and molecular basis of prolonged (~3 weeks) submergence tolerance in rice. Submergence stress of different durations viz. 10 days, 14 days, 18 days and 22 days were imposed in the studied genotypes by completely immersing them in 100 cm of standing water in cemented tanks. Plant survival and other physiological and biochemical parameters *viz*. plant height, elongation ability, survival rate, total chlorophyll and carotenoid content, leaf gas film thickness, leaf porosity and density were recorded by taking out ~10 pots after each of the stress duration period.

The salient findings of this experimentation are summarized below:

1. Out of the 8 studied genotypes, AC42088, AC42087 and AC1303 have significantly higher submergence tolerance ability than FR13A, especially when the stress period went beyond two weeks. Understanding the mechanism of tolerance in these genotypes can significantly improve flood-tolerant rice.
2. The genotypes viz. AC42088, AC42087 and AC1303, possessing beyond two week of submergence tolerance, had invariably lesser EA than our tolerant check FR13A at any point during the entire period of submergence stress.
3. The genotype AC1303 had the least plant height after submergence at the end of entire duration of submergence stress, thereby showing least internode elongation after three weeks of stress, which might be one of the reasons for its greater survival under prolonged submergence.
4. Among the eight rice genotypes, AC1303 was found to possess highest leaf

gas film thickness both on adaxial and abaxial sides (29.39 µm) before submergence, followed by AC 42088 (29.19 µm). Also, significantly greater leaf gas film retention ability was observed in the genotypes like AC1303 followed by AC42088, AC42087, which could able to retain their gas film even up to 8 days after submergence.

5. The leaf tissue porosity was found to be highest (50.95 %) in FR13A, followed by AC 1303 (49.09 %) and AC 42088 (47.83 %) under control condition. Interestingly, among the studied genotypes both FR13A and AC1303 showed least reduction in tissue porosity during our course of study.
6. The leaf blade surface was found to be hydrophobic initially on all genotypes as the contact angle was found to be >125° in genotypes like AC1303, AC42088 and AC42087 and significantly lower in susceptible genotypes like IR42 (112°) and Swarna (116°). It was gradually reduced with duration of stress. After, four days of complete submergence leaf contact angle remained 105° in AC1303, while it was 81° in FR13A and became 0 in Swarna and IR42.
7. Similarly, the epicuticular wax content also varied in the leave of studied genotypes. It was highest in AC1303 (21.61 µgm), which is comparatively more than that of the tolerant check varieties, like: FR13A (13.12µgm) and Swarna *sub-1* (12.7 µgm The variety AC1303 performed well even after 7 days of complete submergence showing 6.69 µgm of wax content, which might help this genotype to survive prolonged submergence stress of beyond two weeks.
8. The susceptible genotypes (IR42, Swarna and AC 38575) exhibited rapid decline in total chlorophyll content under stresses condition as compared to tolerant check (Swarna Sub-1 and FR13A) varieties. The genotypes showing better survival rate than FR13A or Swarna Sub-1 under longer duration of submergence stress showed lower rate of chlorophyll reduction under submergence stress.

9. A gradual reduction in starch content was observed in all the genotypes under submergence stress. The the genotype AC1303 had lowest (3.34%) per day reduction in starch content followed by AC42088 (7.34%) and AC42087 (8.54%), which showed comparatively lower rate of reduction that FR13A and Swarna-Sub1.
10. Profiling of these genotypes based on *SUB1* QTL specific SSR marker showed Swarna lies as outlier with respect to all the other genotypes including Swarna Sub-1. The tolerant genotypes formed different sub-clusters and AC1303 was distinct from the rest in UPGMA and Jaaccard's similarity coefficient analysis.
11. The expression analysis of *SUB1A* gene showed that massive induction of expression was observed in FR13A, but it was only ~135-fold in susceptible genotype IR42. Surprisingly, the expression of *SUB1A* gene was intermediate in AC1303, a genotype having better prolonged submergence tolerance ability than FR13A.

5.2. CONCLUSIONS

I. From the present study, we found a few potential genotypes viz. AC42088, AC42087 and AC1303, which possess submergence tolerance ability of three weeks, a trait absent in most submergence tolerance genotype (FR13A) reported till date.

II. Although *SUB1* gene specific SSR markers suggested genetic similarity of these genotypes (with respect to presence of *SUB1* QTL) with known submergence tolerant lines like FR13A and Swarna-Sub1, but surprisingly the expression profile of *SUB1A* gene is much less in them as compared to FR13A.

III. The above results give a clear indication that there might some other factors apart from SUB1 QTL in these genotypes contributing to their exceptional submergence tolerance behavior.

IV. Possibly, leaf traits like gas film retention ability, tissue porosity and underwater maintenance of leaf hydrophobicity are the additional factors contributing to prolonged submergence tolerance ability in these genotypes.

5.3. SUGGESTIONS FOR FUTURE RESEARCH WORK

The present study conclusively proves the superior submergence tolerance ability in the genotypes viz. AC42088, AC42087 and AC1303 as compared to know submergence tolerant checks like FR13A and Swarna-Sub1. In the event of complete submergence beyond 14 days, the survival rate of both FR13A and Swarna-Sub1 reduces significantly. But these genotypes can withstand complete submergence up to a period of three weeks without much decline in survival rate. Genotypic profiling based on *SUB1* gene specific SSR markers were able to cluster these genotypes with either FR13A or Swarna-Sub1 (except, AC1303), both of which known to possess *SUB1* QTL. It suggested that these genotypes may also possess the *SUB1* QTL identified for submergence tolerance in rice. But interestingly, the expression pattern of *SUB1A* was more than double in FR13A (~2700-fold) as compared to AC1303 (~1200-fold). This suggests there might be some SUB1A independent factors contributing to prolonged submergence tolerance ability in these genotypes. A comparatively dissection of different submergence tolerance strategy may give an insight of the most important components of tolerance mechanism in rice. A detailed study on each components of submergence tolerance strategy might reveal the additional factors responsible for such prolonged submergence tolerance behavior in these genotypes. Besides, a thorough mechanistic study can lead to novel gene/QTL identification from these genotypes which might be useful for transferring prolonged submergence tolerance genes in cultivated rice background.

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APPENDIX-A

Table: Agro-meteorological data (Weekly) of Cuttack during the period of experimentation from Jan 1, 2019 to March 30, 2019

Date	Maximum Temperature (°C)	Minimum Temperature (°C)	Rainfall (mm)	Relative Humidity 1 (%)	Relative Humidity 2 (%)	Wind.Velocity (Kmph)	Evaporation (mm)	Sunshine (Hrs)
January 2019 1-7	29.1	10.6	0	86.42	36.42	2.32	2.24	4.21
8-14	29.37	12.04	0	88.28	53.14	2.52	2.38	3.78
15-21	27.35	12.52	0	72.57	54.28	2.88	1.92	5.7
22-31	27.2	15.27	0	83	46.4	2.38	2.09	4.5
February 2019 1-7	30.1	16.15	0	78	45.71	3.44	1.92	6.347
8-14	31.01	16.12	0	79.71	43.57	3.82	2.01	5.84
15-21	31.14	17.25	0.42	89.57	43.71	2.48	1.87	7.48
22-28	33	20.18	2.6	90	58.85	2.74	1.55	5.82
March 2019 1-7	30.64	21.04	0.85	80.28	64.57	3.28	1.85	5.12
8-14	33.48	22.58	0	80.57	58.14	2.14	2.27	6.71
15-21	32.34	21.58	1.4229	77.71	52.71	3.48	2.24	5.44
22-31	35.4	23.9	0.0	86.0	53.0	3.5	3.4	6.9

RESUME

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