Effects of fluoride on the morphological and biochemical parameters of rice and its FEX gene expression.

Dissertation submitted in partial fulfillment for the degree of

Master of Science in Biotechnology

Submitted By

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Deemed to be University U/S 3 of UGC Act, 1956

CERTIFICATE

This is to certify the dissertation entitled "***Effects of fluoride on the morphological and biochemical parameters of rice and its FEX gene expression***" ssubmitted by ***Alice Arati Anthony*** in partial fulfillment of the requirement for the degree of Master of Science in Biotechnology, KIIT School of Biotechnology, KIIT University, Bhubaneswar bearing Roll No. **1661003** & Registration No. *'**1000037069**'* is a *bona fide* research work carried out by her under my guidance and supervision from '8th January 2018'to '17th May 2018'.

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Deemed to be University U/S 3 of UGC Act, 1956

CERTIFICATE

This is to certify that the dissertation entitled "***Effects of fluoride on the biochemical parameters of rice and its FEX gene expression***" submitted by '***Alice Arati Anthony***, *Roll No.**1661003**, Registration No.**1000037069***' to the KIIT School of Biotechnology, KIIT University, Bhubaneswar-751024, for the degree of Master of Science in Biotechnology is her original work, based on the results of the experiments and investigations carried out independently by her during the period from '8th January 2018'to '16th May 2018' of study under my guidance.

Further, it is also to certify that the above said work has not been previously submitted for the award of any degree, diploma, or fellowship in any Indian or foreign University.

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Assistant Professor
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DECLARATION

I hereby declare that the dissertation entitled "***Effects of fluoride on the morphological and biochemical parameters of rice and its FEX gene expression***"submitted by me, for the degree of Master of Science to KIIT University is a record of *bona fide* work carried by me under the supervision and guidance of '***Dr. Sasmita Mohanty***', ***Associate Professor***,***KIIT School of Biotechnology, KIIT University, Bhubaneswar.***

Date: ***Alice Arati Anthony***

Place:

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Date:
Place: Bhubaneswar **Alice Arati Anthony**

CONTENTS

ABBREVIATION

F	Fluoride
NaF	Sodium Fluoride
Os	*Oryza sativa*
At	*Arabidopsis thaliana*
ROS	Reactive oxygen species
SOD	Superoxide dismutase
gm	Grams
L	Litre
ml	Milliliter
µl	Microliter
Mg	Microgram
ppm	Parts per million
ºC	Degree celsius
%	Percentage
Temp	Temperature
Mins	Minutes
Conc.	Concentration
Chl	Chlorophyll
DNSA	3,5-Dinitrosalicylic acid
FEX	Fluoride Export proteins
MSA	Multiple Sequence Alignment

cDNA	Complementary deoxyribonucleic acid
MCT	Microcentrifuge
DEPC	Diethyl pyrocarbonate
mRNA	Messenger Ribonucleic acid
PCR	Polymerase Chain Reaction
RT-PCR	Real Time-Polymerase chain reaction
RNA	Ribonucleic acid
RT	Reverse transcribed
TAIR	The Arabidopsis information resource
PIR	Protein Information Resource
MEGA	Molecular evolutionary genetic analysis
TM	Trans membrane

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ABSTRACT

Fluoride (F) toxicity in plants is caused due to significant accumulation of F in the crops irrigated with F-contaminated water, which causes inhibition of the growth and development and reduction in the photosynthesis in many plant species. F in water, soil, gases and dust effects the plants and vegetation altering the leaf physiology. Signs of F injury to plants may be acute or chronic depending upon the duration and frequency of F exposure. Fluorine is an abundant element and is toxic to organisms from bacteria to humans, but the mechanisms by which eukaryotes resist fluoride toxicity are unknown. The phenotypic and physiological effects of plants in response to F varies from plant to plant and even at cultivar level and studies in this regard are scanty.

In the present study, we evaluated the effect of high fluoride on the germination, growth and other biochemical characteristics of *Oryza sativa* var. CR Dhan 304 seedlings. Additionally we carried out *in silico* analysis of Rice FEX orthologs and expression analysis of rice FEX gene in high fluoride conditions. Results suggest that exposure of high F to this variety of rice is detrimental and this variety may not be recommended for cultivation in high F areas.

Chapter 1:

Introduction

Fluorine is a highly toxic and corrosive gas, light yellow-green in colour with a pungent smell. It was discovered by a French scientist Henri Moissan in the year 1886 for which he was awarded the Nobel Prize in 1906.It is the 13^{th} most abundant element of the halogen group available on the earth's crust having atomic number-9 and molecular weight-19.Fluorine is so active chemically that it is found in nature only in combination with other elements.

Fluoride, the compound form of fluorine accounts for about 0.3g/kg (0.06 to 0.009%) of the earth's crust. Fluoride (F) is a phytotoxic air pollutant originating from geological deposits and is found in natural waters at different concentrations. Fluorine compounds are widely distributed, they are found in varying amounts in minerals, rocks, gases from volcanoes, and fumaroles, soil, plant and animal tissues and certain waters.

The principal fluorine bearing minerals which are used in large scale industrial processes are: fluorite or fluorspar (CaF_2), cryolite (Na_3AlF_6), apatite ($3Ca_3(PO_4)_2CaF_2$) and sedimentary phosphate rock. Fluorspar is used extensively as a flux in smelting of metals and in the ceramic industries, while cryolite is used in the manufacture of aluminum. Elemental phosphorus and phosphatic fertilizers are manufactured from the deposits of phosphate rocks containing fluorides. Fluorine compounds are also used in the etching of glass, in insecticides and as catalysts in many chemical processes. As fluorides are emitted by such industries, elevated levels of fluoride concentration in plants and soils are found near these industrial emission sources and there are reports of vegetation damage in such locations. Many municipal water sources inject fluorine at 1 ppm as an additive to prevent tooth decay. Use of this water for irrigation can also result in toxicity symptoms on sensitive plants, like necrosis on the tips and margins of the leaves that spreads inside. The occurrence of F is also influenced by high ambient temperature, alkalinity, calcium, and magnesium in the ground water.

Fluoride in small amounts is necessary for plant growth; but when in high concentrations, can cause damage to the plants and also to the environment. Fluoride is

mainly absorbed by the plant roots from the soil and is transported through the xylematic flow to the transpiratory organs like the leaves. The accumulation of fluoride in decreases in the order roots>leaf>stem>seed. The initial and visible symptoms of F toxicity to plants are the commencement of necrosis at the tips and margins of the leaves. Continuous and prolonged F exposure causes adverse affects on the growth and yield of plants. Fluoride causes significant impacts on health among people through drinking-water. Excessive exposure to fluoride in drinking-water, or in combination with exposure to fluoride from other sources, gives rise to a number of adverse effects, ranging from mild dental fluorosis to crippling skeletal fluorosis in humans. Crippling skeletal fluorosis is a significant cause of morbidity and no treatment is known so far for F toxicity.

Atomic number	9
Atomic mass	18.998403 $g.mol^{-1}$
Electronegativity according to Pauling	4
Density	$1.8*10^{-3}$ $g.cm^{-3}$ at 20°C
Melting point	-219.6 °C
Boiling point	-188 °C
Vanderwaals radius	0.135 nm
Ionic radius	0.136 nm (-1); 0.007 (+7)
Isotopes	2
Electronic shell	[He] $2s^2 2p^5$
Energy of first ionisation	1680.6 $kJ.mol^{-1}$
Energy of second ionisation	3134 $kJ.mol^{-1}$
Energy of third ionisation	6050 $kJ\ mol^{-1}$
Standard potential	- 2.87 V
Discovered by	Moissan in 1886

Figure 1: Chemical properties of fluorine

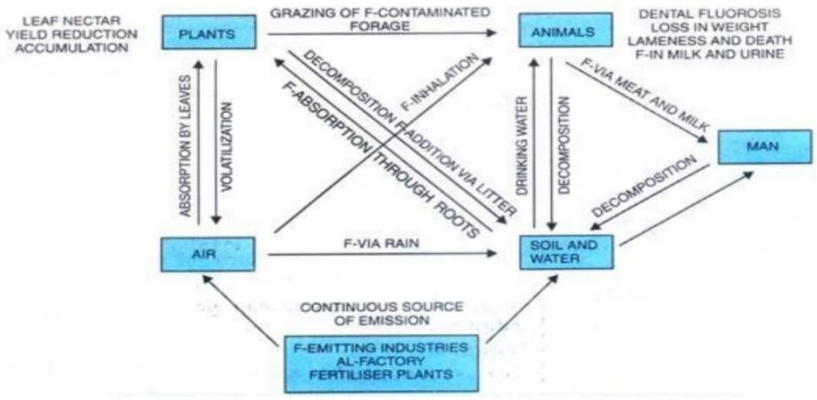


Figure 2:Effects of fluoride on plants, animals and humans.

High fluoride concentrations are prevalent in ground waters from calcium-poor aquifers and in areas where fluoride-bearing minerals are common and have been reported from many countries including India (Fawell *et al.*, 2006). In India more than 66 million people are at the risk of developing fluoride toxicity due to ground water contamination (> 1 mg/L as prescribed by WHO) is widespread in the arid western states of Rajasthan, Gujarat and Punjab to southern states of Andhra Pradesh, Karnataka and Tamil Nadu. As per the records of the Central Ground Water Board (CGWB), Government of India, in Odisha, F contamination is wide spread with 27 out of 30 districts facing the grave challenge (Mahapatra, 2007).

Rice is the staple food of over half of the world's population including India, India is the second leading producer of rice in the world after China. The annual rice production in India is is around 85-90 million tons and annual consumption is 85 million tons. In Odisha rice is synonymous with food, about 69% of the cultivated area is covered by rice and it is the major crop covering about 63% of total area under food grains (Das, 2012) Fluoride contaminated groundwater used to irrigate crops especially paddy rice for long time results in elevated soil fluoride levels, which is a growing concern regarding accumulation of fluoride in rice grown in these soils. Fluoride accumulated in rice straw at high levels will directly affect the health of the feeding cattle and also indirectly to human health through contaminated meat and milk. Although F accumulation in the human body occurs through F-contaminated drinking water, substantial amounts of F are ingested through cereals and vegetables cultivated with F-contaminated irrigation water (Poureslami *et al.*, 2008; Gupta *et al.*, 2009).

In this context, the present study was carried out with the following objectives:

1) To study the effect of high fluoride on the germination and seedling growth (shoot and root length) of rice
2) To study the chlorophyll and carotenoid content, soluble sugars and the lipid levels of rice seedlings exposed to high fluoride
3) The accumulation of fluoride in different parts of the plant i.e, shoot and root
4) *In silico* analysis of Arabidopsis FEX genes and its Rice orthologs
5) Expression analysis of rice Fluoride Export proteins (FEX) in high fluoride conditions

Chapter 2:

Review of Literature

Rice (*Oryza sativa*) is a major dietary staple of nearly half the world's population .About 95% of this cereal is produced and consumed in South East Asian countries, including India. It is estimated that more than half the population of India subsists on rice. Rice is a model monocot system, the world's most important agricultural species. The genus Oryza belongs to subfamily *Oryzoideae* of family *Gramineae*. It has two cultivated species of rice, *Oryza sativa L*. grown worldwide and *Oryza glaberrima* Steud grown in parts of West Africa and more than 20 wild species. About 80% of the calories is derived from rice. Rice is produced in a wide range of locations and under a variety of climatic conditions, from the wettest areas in the world to the driest deserts. It grows in the tropics, subtropics, semi-arid tropics and temperate regions of the world. Rich in nutrients and vitamins and minerals, it is an excellent source of complex carbohydrates. It is the backbone of livelihood for millions of rural households and plays a vital role in the country's food security, so the term "Rice is life" is most appropriate in Indian context.

Abiotic stress is a major factor that limits the plant growth and productivity. Abiotic stress can be defined as any environmental condition which can reduce the growth, survival, and/or fertility of plants. The environmental conditions leading to stress includes UV and visible radiations, high and cold temperatures, water stress, nutrient deficiency during various developmental stages of the plant etc. Abiotic stress is one primary cause of crop loss worldwide and can cause average yield loss of more than 50% for major crops. Plants can resist the abiotic stress through different distinct mechanisms. The tolerance and susceptibility to abiotic stress is very complex.

Fluoride Toxicity

Fluoride an important environmental contaminant, enters into plants via roots and is subsequently translocated to the transpiratory organs of the plant. Fluoride toxicity is a major abiotic stress and its negative effects on crop productivity and growth rate is well known. Various physio-biochemical changes have been reported in plants due to F toxicity which disturb their metabolism causing growth inhibition and ultimately plant death.

Fluoride in Soil

Agricultural soil rich in F is common due to the long term accumulation of F from various sources like the industrial wastes and extensive usage of phosphate fertilizers and F containing pesticides. As the soil pH decreases below 5.6, the concentration of soluble fluoride increases and hence there is an increase in fluoride available to the plants. Certain soil minerals have the capacity to fix fluoride in a form that is unavailable to plants. Calcium is one such component, but other constituents such as aluminum and iron (iii) hydroxides have also been shown to be important.

Effect of Fluoride in plants

Some plants accumulate F and are able to grow even at high concentrations of F without showing any signs of damage while several other plants sustain injuries at even low concentrations of F. The rate at which the symptoms appear in the plants depends upon various environmental factors like concentration of F, length of exposure, age, genotype of plants and meteorological conditions.

Fluoride treated plants show reduced growth parameters i.e. seedling germination percentage, length of shoot and root, biomass accumulation etc. Failure of germination may be due to reduced water uptake, inhibited cell division, and enlargement in embryo and/or overall decrease in the metabolic activity associated with these processes. The decrease in shoot and root length is because of unbalanced uptake of nutrients by the plants in the presence of fluoride. At molecular level F causes DNA damage which effects the gene expression which is further responsible for particular amino acid formation leading to change in the growth parameters (Agarwal and Khan, 2016).

F inhibits photosynthesis and other processes. The rate of photosynthesis is mainly affected by reduced chlorophyll synthesis or by degradation of the ultrastructure of the chloroplasts and inhibition of the Hill reaction (Baunthiyal and Ranghar, 2014). Due to the high electronegativity of F the chlorophyll molecule gets destroyed and the disintegration of chloroplast gets accelerated. F inhibits the photosystem-II (PS-II) electron transport rate followed by subsequently increasing the rate of photosystem-I (PS-I) indicating the possibility of state transitions responsible for the mechanism of

fluoride toxicity (Baunthiyal and Ranghar, 2014). It was shown that with increase in the application NaF on watermelon *(Citrullus lanatus)* there was decrease in the photosynthetic pigments; Chlorophyll-a, chlorophyll-b, total chlorophyll and carotenoids (Ram *et al*., 2014). This decrease may be due to the inhibition of chlorophyll biosynthesis. The reduction of carotenoids may be possibly due to the F induced stress inhibition of carotenoids formation in plant cells.

The mechanism of respiration is also inhibited by F which may be linked to inhibition of the respiratory enzymes and stimulation may be linked to an uncoupling of phosphorylation. The respiratory enzymes like succinate, malate, and NADH dehydrogenases are all F sensitive, with succinate dehydrogenase, in the presence of phosphate, being the most sensitive (Baunthiyal and Ranghar, 2014).

Plants have antioxidants, defence mechanisms consisting of enzyme catalase, peroxidases, superoxide dismutase and non enzymatic constituents. These antioxidant systems consistently protect the cell from lipid peroxidation caused by F exposure, which suggests that oxidative damage is the major mode of action of F. F induced oxidative stress causes increased production of the reactive oxygen species (ROS). ROS triggered by fluorine compounds include superoxide radical, hydroxyl radical and hydrogen peroxide which cause damage to the biomolecules such as membrane lipid proteins, lipids, plant pigments, enzymes and nucleic acids. Superoxide dismutase (SOD) eliminates superoxide radicals from the cell environment and prevents the formation of toxic ROS and their derivatives. Exposure of F was shown to increase the SOD activity in the F treated seedlings of *Oryza sativa*. This may be related to the increased metabolic status or its biosyntheisis in F affected tissues and can be considered as a positive feedback mechanism. (Chakrabarti and Patra, 2015). The catalase activity is reduced in the fluoride treated paddy plants due to inhibition of the enzymatic system or a change in the actual catalase concentration in the fluoride treated tissues compared to the non treated tissues. Reduction may be due to the hydroxyl ions (OH^-) attached to the iron atoms in catalase compounds are replaced by low molecular weight anions in sufficient concentration leading to inhibition.

Fluoride effects on biomolecules

Carbohydrates:

High dose of F resulted in reduced sugar accumulation in *Oryza sativa* seedlings (Ram et al., 2014). F inhibits photosynthesis, reduces the activities of invertase and amylase and increases oxidative stress which may be responsible for the reduced sugar accumulation. F severely inhibits the activities of more than 300 enzymes by removing a cofactor Mg^{2+}. Moreover the partitioning of the photo assimilates for sucrose and starch synthesis, is directed by F- mediated pyrophosphate (PPi) accumulation. Total soluble sugars may increase under F stress in plants and its level is directly related to the stress factors. Fluoride stress increased the soluble sugars content in *Oryza sativa* and *Cicer arietinum (*Chakrabarti *et al*., 2017). The increased sugars may act as osmolytes which increase the water potential of the seedlings for survival under F stress since fluoride inhibits root water transport.

Lipids:

The oxidation of the poly unsaturated fatty acid (PUFA) fractions of lipids in the plasma membranes is the most damaging and key process known to occur due to F toxicity (Chakrabarti *et al*., 2017). The peroxidation reaction of this macromolecule includes three important steps viz., initiation, progression, and termination. Peroxidation of membrane lipids is initiated when a OH radical abstracts one hydrogen atom from a PUFA. The lipid peroxidation reaction exacerbates the oxidative damage through the production of lipid-derived secondary free radicals that themselves can react with and damage both proteins and DNA. PUFAs get oxidized from ROS and generate a number of cytotoxic by-products namely malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE) and hydroxyl and keto fatty acids. Significant change in in the pace of lipid peroxidation reaction has been seen in *Helianthus annuus* (Sunflower)with increasing F and time of exposure (Saleh and Abdel-Kader, 2003).

Proteins:

The addition of F reduced the protein content in both a dose and time dependent manner in a variety of seedlings (Datta *et al.*, 2012; Gadi *et al.*, 2012). Reduced synthesis, enhanced degradation and usage for energy production are held responsible for this lowering of protein in stressed seedlings (Baunthiyal and Ranghar, 2014). Like lipids, proteins are also prone to ROS attack, which may cause deleterious

modifications via nitrosylation, carbonylation, formation of disulphide bonds and glutathionylation. F has been shown to be involved in the synthesis of misfolded proteins in the endoplasmic reticulum and consequent ROS generation. The expression of genes associated with stress response factors (eg. heat shock proteins--; hsp70), signal tranduction components and apoptosis related proteins were demonstrated to be up-regulated by exogenous F application (Barbier *et al*., 2010).

DNA:

Reduced DNA synthesis has been revealed due to F- toxicity which may be possibly linked to insufficient DNA Polymerase activity. This reduced DNA is also related to decreased RNA and protein synthesis and reduced rate of cell division and elongation (Panda, 2015). Electrophoresis and qRT-PCR studies have demonstrated the rate of DNA damage and the expression of the caspase-3 and -9 genes respectively to be significantly enhanced with exogenous F application (Wang *et al*., 2004; Song *et al*., 2015).

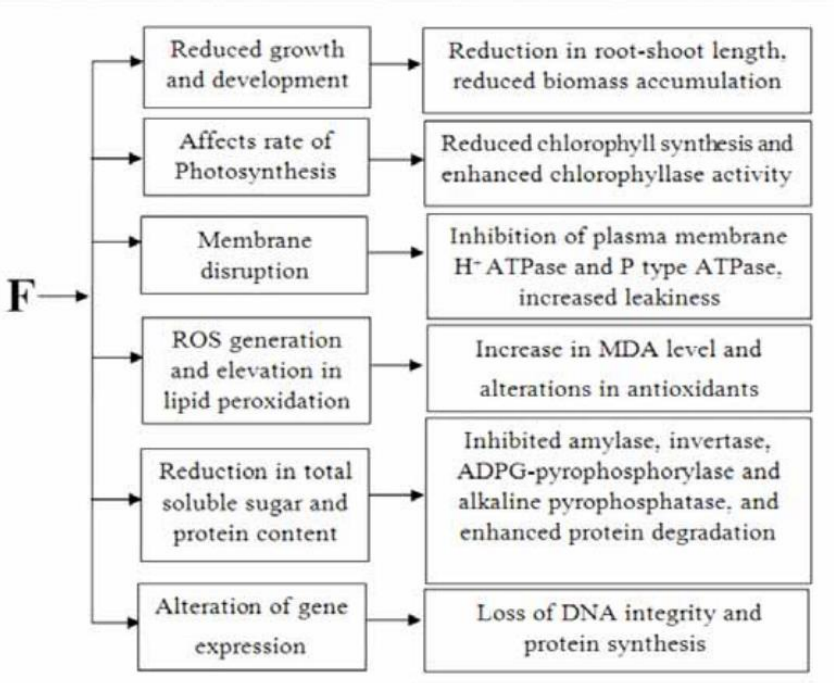


Figure 3: F-induced physiological, biochemical and molecular modifications in plants (Yadu *et al*., 2016).

Mechanism of uptake of fluoride into plants

The uptake of fluoride into plants is mostly via the roots and leaves. Gaseous or particulate F is absorbed through the stomata of the leaves and is less likely to be

absorbed through the cuticle of the leaf (Weinstein and Alscher-Herman, 1982). Uptake of F from the soil is usually by absorption of F in the soil solution through roots by passive diffusion process via xylem tissues through apoplastic and symplastic pathways into the shoots. F can also transport through the biological membranes via non ionic diffusion of Hydrogen Fluoride (HF). HF penetrates into the cell membranes much faster than the dissociated F ion. It's membrane permeability is 5-7 orders of magnitude above that of F ion. Fluoride uptake is also influenced by elements like Aluminium (Al) and Boron (B). It had been shown that the uptake of fluoride had increased in tea and other plant species by supplying Al in nutrient solution or soil (Ruan *et al*., 2003). Fluoride gets transported as Al-F complexes in the xylem and dissociates after reaching the leaves (Nagata *et al*., 1993).

Figure 4: Transport of fluoride by plants.

Recent Studies:

The effects of fluoride on plants have been studied by various researchers. According to Gupta *et al.* (2009) the shoot, root and dry weight decreased with increasing concentration of NaF. The chlorophyll content also decreased with increasing concentration of NaF but the reducing sugars was found to initially decrease and then increase with increase in NaF.

According to the reports of Chakrabati *et al.* (2015) chlorophyll, carotenoid, free sugars and catalase activities decreased with increase in fluoride treatment but the peroxidase and SOD activities as well as free amino acids content had increased.

In 2013, Chakrabarti and Patra showed that the fluoride uptake and SOD activity both increased with increased F exposure in order radish > coriander > spinach > mustard. Their results also pointed out that plant species tolerant to F toxicity influence higher antioxidant SOD activity, which the plant cells may have adapted to diminish the harmful effects of ROS generated during F stress.

In 2009, Gupta and Banerjee reported that the accumulation of fluoride decreased in paddy in the order: root > leaf > stem > seeds, indicating maximum accumulation in the root part of the plant.

Fluoride Exporters (FEX)

Because of the ubiquitous presence of F in nature and its toxic effects, different organisms evolutionarily have evolved mechanisms of resistance to fluoride. However, little is known about these strategies and pathways. Recently the discovery of fluoride riboswitch class in eubacteria and archaea indicated the cellular mechanism to avoid fluoride toxicity. Riboswitches are metabolic binding RNA structures which are present in bacterial messenger RNAs controlling gene expression. The members of the riboswitch class which was discovered are selectively activated by fluoride but turn down other small anions, including chloride (Baker *et al*., 2012). The genes includes two distinct families of membrane proteins, the CLC^F which is a F^-/H^+ transporter that belongs to the CLC superfamily and CrcB, which are small membrane proteins that help in fluoride export across the cell membrane (Stockbridge *et al*., 2015). The *crcB* gene initially identified in E.coli had been involved in chromosome condensation and camphor resistance and encodes a small protein (127 amino acids) having four predicted transmembrane domains. The crcB later renamed as Fluc are ion channels with greater selectivity i.e >10000 fold for fluoride over chloride. Flucs are antiparallel dimers forming two active fluoride pores, each monomer comprising four transmembrane alpha helices. The functional unit may be different in different organisms, a homodimer or heterodimer but the transmembrane topology is highly conserved (Li *et al*., 2013).

It has been proposed that Fluc uses aromatic and hydrogen bond donating amino acids within its pores to transport fluoride ions. Highly conserved phenylalanines are located in TM3 that face into the pore and adopt a side-to-face ‘box’-like arrangement for interaction with fluoride. The residues facing the pore in TM4 are different

hydrogen bond donars of low sequence identity, every fourth residue in TM4 varies between N, S, T, H or Y, suggested to give a polar track needed for recognition of fluoride ions. The two pore model of Fluc was confirmed by mutagenesis of the four highly conserved phenylalanine residues located in the pore (Stockbridge *et al.*, 2015).

To determine whether the eukaryotic homologs of crcB are involved in fluoride homeostasis, Li *et al.* (2013) used genetic approaches in the filamentous fungus *Neurospora crassa*, the budding yeast *S. cerevisiae*, and the pathogen *Candida albicans* and demonstrated that CrcB proteins play a role in fluoride resistance in eukaryotes. Based on these results, they proposed renaming these genes as FEX, for fluoride exporters (Li *et al*., 2013).

The word FEX i.e fluoride Export protein is used to refer to the fluoride channels conserved in eukaryotes. Eukaryotic FEX proteins have only one functional pore unlike the two functional pores in bacterial Fluc but both the domains are retained by the eukaryotic channels. It has been identified that Pore II was functional in fluoride transport, the Pore I is left dysfunctional due to the evolutionary drift. However the overall architecture is conserved from bacteria to eukaryotes. Mutational studies of FEX along with the physiological and structural studies of Fluc provides an understanding of the mechanism of F selectivity within FEX. Fluoride being highly electronegative and having small atomic radius is a good hydrogen bond acceptor. Conserved residues with hydrogen bonding potential are speculated from the Fluc structure to line Pore II of FEX and are crucial for fluoride tolerance in yeast. These residues imitate the chemically preferred hydrated state of fluoride ion enabling the desolvation of fluoride from aqueous solution. Two sets of highly conserved Phe residues are also important for FEX to function.

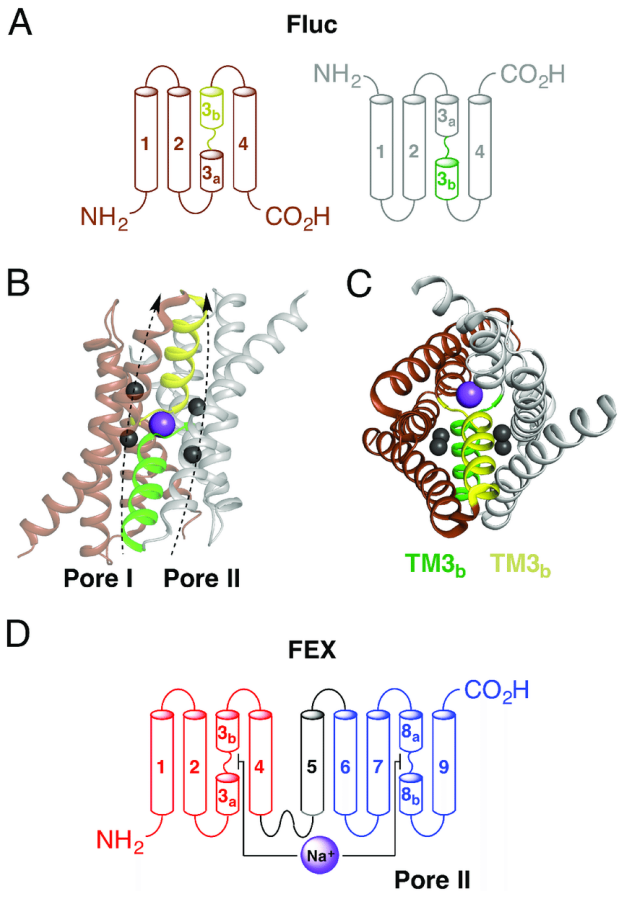


Figure 5: Structural arrangement of fluoride export proteins (Berbasova *et al.*, 2017).

(A) Topology model of bacterial Fluc protein. (B) Tertiary structure of Fluc-Bp (PDB ID: 5FXB) showing two pores with fluoride (dark grey) ions. Sodium ion (purple) is in the middle of the protein dimer. Two monomers are coloured brown and light grey. (C) Top view arrangement of transmembrane helices. Helices TM3b from different monomers (yellow and green) separate two pores and have residues belonging to two different pores but located on the same helix. (D) Topology model of eukaryotic FEX protein with proposed interactions between TM3 and TM8 based on Fluc crystal structure.

FEX genes are widespread across all three domains of life, suggesting that these proteins have an important function in diverse organisms. Sequence alignment suggests that there are FEX-like proteins in many plants and some animals though none of them have been characterized. The FEX-like protein from the model plant *Arabidopsis thaliana* currently annotated as ORF At2g41705.1 has only 27% sequence identity to yeast FEX, but it includes all the residues identified as importantfor fluoride tolerance (Berbasova *et al.*, 2017). Blast searches using *Saccharomyces cerevisiae* FEX have

identified FEX homologs in important agricultural plants- corn, rice, grapes, oranges and cucumber. Almost all plants whose genome is available having a predicted open reading frame encodes a FEX homolog. In contrast to two functional pores in bacterial Fluc, eukaryotic FEXproteins retain only one functional pore, yet the eukaryotic channel retains both domains. Out of the two,pore II is active andanother pore is vestigial in eukaryotic fluoride channels (Berbasova *et al.*, 2017; Figure 6). In spite of this evolutionary degeneration of one of the pores, the overall architecture of the channel is conserved from bacteria to eukaryotes(Berbasova *et al.*, 2017).

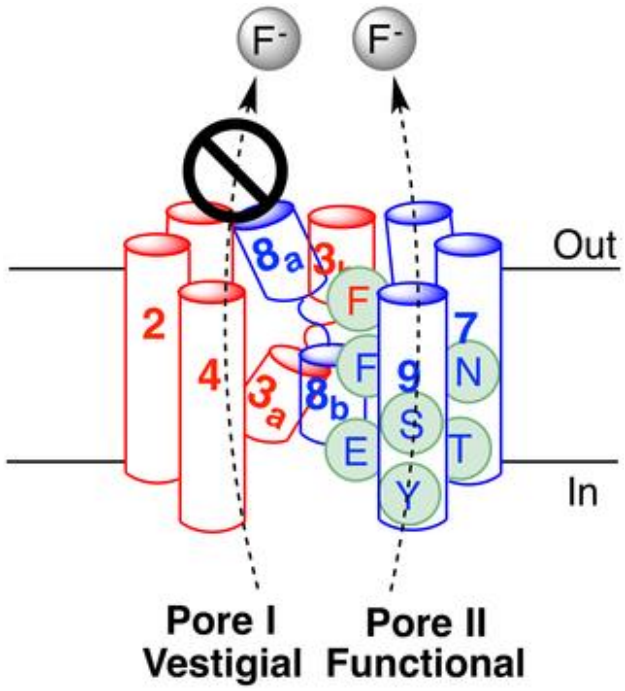


Figure 6: The helices from both domains forming functional and vestigial pores (Berbasova *et al*., 2017).

Chapter 3

Materials And Methods

- **Materials and their sources:**

All the chemicals and reagents required for the experiments were ordered from Himedia Laboratories, India
Biotech instruments, USA- Nanodrop
Eppendorf, Germany- Centrifuge
Chromous Biotech, India- Gel electrophoresis apparatus
BIO-RAD- Thermal cycler and CFX Connect Real-Time System.
IDT (Integrated DNA technologies)- Primers

Sample collection:

The rice seeds were obtained from National Rice Research Institute, Cuttack. It was of variety CR Dhan 304. Its maturity period is 125-130 days and it is suitable for growing in irrigated medium land ecology. This variety was released in the year 2014 to be grown in the states of West Bengal and Odisha.

Seed sterilisation and plating:

- The rice seeds were soaked in distilled water for few hours.
- They were sterilised by stirring in 0.1% Mercuric Chloride for 2 minutes.
- The seeds were then washed by running tap water and then distilled water.
- The seeds were transferred to Petri plates containing germination paper moistened and treated with 0mg/l (control-water), 15mg/l, 30mg/l, 50mg/l and 100mg/l NaF.
- The Petri plates with seeds were kept in dark for 3 days after which the seed germination percentage was calculated and the plates were transferred to plant tissue culture lab whose temperature was maintained at 27 ^{o}C +/- 5 ^{o}C and humidity was maintained at 78 ^{o}C +/- 5 ^{o}C.
- After 15 days the root and shoot lengths were taken, biochemical parameters like chlorophyll content, reducing sugars, lipids and fluoride accumulation was also analyzed.

Methodology:

- **Germination percentage(%)**

The germination percentage is the average number of seeds germinated during the 3 days kept in dark. It can be calculated by the following formula:

Germination (%) = (Number of seeds germinated/Total number of seeds in the plates)*100

- **Shoot and root length**

The shoot and root lengths were measured using a cm scale after 7^{th} and 15^{th} days.

- **Estimation of Chlorophyll and Carotenoids in leaves**

- 100 mg of control and NaF treated leaves were taken and homogenised with 80% acetone in a pre chilled mortar and pestle and the volume was made upto 10 ml by re-extracting with acetone.
- The samples were taken in a 15ml falcon tube and centrifuged at 10000 rpm for 5minutes at 4 degree celsius.
- The supernatant was taken and transferred to a new tube. The absorption of the extracts were measured spectrophotometrically at 663nm, 645nm and 440nm for chlorophyll a, chlorophyll b and carotenoids respectively.
- The concentration of chlorophyll a, chlorophyll b, total chlorophyll and carotenoids were calculated by using the below formula according to Porra *et al.* (1989) and Holm (1954):

Chlorophyll a (μg/ml)= $12.25 * A_{663} - 2.55 * A_{645}$

Chlorophyll b (μg/ml)= $20.31 * A_{645} - 4.91 * A_{663}$

Total chlorophyll (μg/ml)= $17.76 * A_{645} + 7.34 * A_{663}$

Carotenoids (μg/ml)= $4.69 * A_{440} - 0.267 *$ total chlorophyll

➢ **Estimation of reducing sugars in leaves:**

The extraction and estimation of reducing sugars was done by the method of Miller (1972) with modifications.

Preparation of DNSA reagent and Rochelle salt solutions:

- 1 gm of 3,5- Dinitrosalicylic acid (DNSA) and 50 mg Sodium sulphite were dissolved in 1% NaOH (1 gm in 100ml distilled water).
- 40 gms of Sodium Potassium Tartarate (Rochelle salt) was dissolved in 100 ml distilled water.

Procedure:

- 0.1 g of control and NaF treated leaves were taken and homogenised separately in hot distilled water and taken in falcon tubes and volume was made upto 5 ml by re-extracting the samples with hot water.
- The samples were centrifuged at 5000xg for 15 minutes at room temperature. Then the supernatant was taken and transferred in a fresh falcon tube.
- 1 ml of supernatant was taken from each of the samples (control as well as the treated) in 5 different test tubes marked as 0 mg/l, 15 mg/l, 30 mg/l, 50 mg/l and 100 mg/l to these 3 ml of the prepared DNSA reagent was added and the test tubes were boiled for 5 minutes in a boiling water bath.
- Then 1 ml of Sodium Potassium tartarate i.e Rochelle salt was added to each of the test tubes.
- The test tubes were allowed to cool to room temperature and the absorption was measured at 530 nm in a spectrophotometer.

➢ **Estimation of Lipids in leaves:**

The extraction and estimation of lipids in NaF non-treated and treated leaves was done following the protocol of Folch *et al*. (1957).

- 0.1 g of leaves were homogenised separately with chloroform: methanol (2:1) and transferred in a falcon tube and the volume was made upto 5 ml by re-extracting.
- The whole mixture was then mixed by vortexing.
- The homogenate was then centrifuged at 5000 rpm for 5 mins to recover the liquid phase.
- The liquid phase was transferred to a new falcon tube, it was washed with 1 ml of 0.9% NaCl solution.

- After vortexing for some seconds, the mixture was centrifuged at low speed i.e 2000 rpm for 2 minutes to separate the two phases.
- The upper phase was removed carefully without mixing the whole preparation and the lower phase containing the lipids was evaporated in boiling water bath till the chloroform smell disappeared.

Weight of the lipids was calculated as follows:
Initial wt of the empty falcon tube= X gm
Final wt of the falcon tube containing lipid after the solvent evaporation= Y gm
So, final wt of the lipid obtained= (Y-X) gm of lipid

- **Spectrophotometric analysis of fluoride using Spadn's reagent**

The F accumulated in the shoots and root of the F treated rice plant was estimated according to the modified method Hussein and Momani (1989).

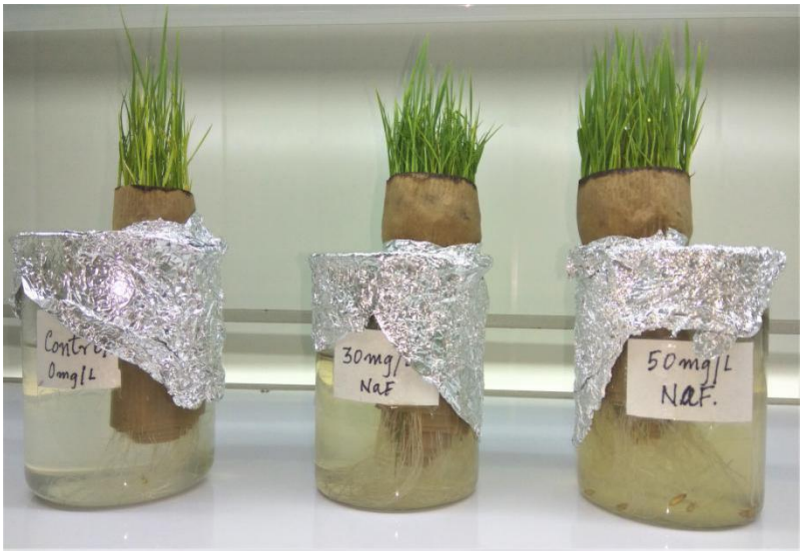


Figure 7: Rice plants grown for 30 days in 0 mg/l, 30 mg/l and 50 mg/l NaF.

Extraction:

The 30 days rice shoots and roots that were grown in 3 different concentrations i.e, 0 mg/l, 30 mg/l and 50 mg/l were taken and fresh weight was calculated separately. Then the leaves and roots were dried in hot air oven for 48 hours. When the shoots and roots were fully dried they were taken in 6 flasks, 3 for the shoots and 3 for the roots and were labelled accordingly. The plant materials were digested inside fume hood over hot plate

using aquaregia. Initially 20 ml of aquaregia was added in all the flasks and allowed to digest, distilled water was being poured to maintain the volume. It was heated until clear solution was obtained.

Preparation of reagent:

SPADNS Solution

[4,5Dihydroxy-3-(p-Sulfophenylazo)-2,7-Naphthalene-Disulfonic Acid Trisodium Salt]: 0.960 g SPADNS reagent was dissolved in 500 mL water.

SPADNS Mixed Reagent: 0.135 g of ZrOCI.8H_20 was dissolved in 25 mL water and dilute to 500 mL with water. Mix equal volumes of this solution and SPADNS solution to form a single reagent.

Spectrophotometer Zero Reference Solution: 5 mL SPADNS solution was added to 25 mL water, and acidify with a solution prepared by diluting 7 mL conc. HCI to 10 mL with water.

Spectrophotometer Calibration: 5 mL SPADNS mixed reagent was added to 25mL water for the blank standard.0, 1,2,3,4,5,6 mL of the 5 ppm standard fluoride solution was diluted to 25 mL with water to produce dilutions of 0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 ppm, respectively. After mixing, the reference standards, reference solutions were placed in a constant temperature bath for 30 mins. Then the absorbance was recorded in the spectrophotometer within 2 hr at 550 nm. The reference solution was used to set the absorbance to zero. Calibration curve was made by plotting concentration of fluoride versus absorbance.

Analysis of the samples extracted:

5ml SPADNS mixed reagent was added in 6 volumetric flasks. 2ml of the extracted shoot and root samples which was grown at 30mg/l and 50mg/l were added in the volumetric flasks and diluted with water. After mixing the solutions, absorbance was recorded in spectrophotometer at 550 nm.

➢ **Isolation of Total RNA from Rice leaves**

Rice seeds were grown in germination paper placed in Petri plates at 2 different concentrations, 0 mg/l and 50 mg/l. After 15-16 days total RNA was extracted from the plant leaves.

For RNA extraction QIAGEN RNeasy plant mini kit (QIAGEN, USA) was used.

- 100 mg rice leaves grown at 0 mg/l and 50 mg/l were taken separately and grounded in a pre-chilled, autoclaved mortar and pestle using liquid nitrogen until fine powder was obtained.
- The fine powder was then transferred to a 2 ml MCT and 450 µl of RLT buffer (previously added with β-mercaptoethanol, 10µl per 1ml RLT buffer) was added and vortexed for 30 seconds.
- Then the lysates were transferred to a 2 ml QIA shredder spin column placed in a 2 ml collection tube and centrifuged for 2 mins at 14000xg at RT. The flow through was transferred to a new MCT and 99% ethanol (0.5 volume) was added and mixed by pipetting.
- 650 µl of the sample was then transferred to the provided RNeasy spin column in a 2 ml collection tube and centrifuged at 8000xg for 15 seconds. The flow-through was discarded.
- 700 µl of the Buffer RW1 was added to the RNeasy spin column.and centrifuged for 15 s at 8000xg. The flow-through was again discarded and 500 µl of Buffer RPE was added to the RNeasy spin column and centrifuged for 15 s at 8000xg.
- 500 µl of Buffer RPE was again added to the RNeasy spin column and centrifuged for 2 mins at 8000xg.
- RNeasy spin column was placed in a 1.5 ml collection tube, 30-50 µl of RNase-free water was added to the spin column membrane and centrifuged for 1 min at 8000xg to elute RNA.

➢ **Estimation of RNA concentration**

The concentration of the extracted RNA from the two samples were measured using a Nanodrop (Biotech instruments, USA) spectrometer. The concentration was measured by putting 1 µl of the sample.

- **Agarose Gel Electrophoresis**

- Agarose gel electrophoresis was performed after RNA extraction.
- 0.8% agarose gel was prepared by dissolving 0.8 g of agarose in 100 ml 1x TAE (Tris Acetate EDTA) buffer and melting in microwave oven, to it 1µl of Ethidium bromide was added and the solution was poured in the gel container with the comb to form well.
- After the gel had solidified, the comb was removed and the gel was placed in an electrophoresis chamber, 1x TAE buffer was added.
- Then 2 µl of RNA extract of the two samples i.e; control and 50 mg/l were mixed with 2 µl of loading dye separately and loaded in the wells. The gel was then run at 70 volts for approximately 45 minutes.

- **cDNA synthesis**

RNA extracted from the rice leaves subjected to 0 mg/l NaF and 50 mg/l NaF was reverse transcribed to form cDNA. It is a two step precess of PCR cycle: cDNA Reverse Transcription Kit and specific oligonucleotides (Oligo dT) primers were used to form the cDNA from the extracted RNA. Oligonucleotide primers selectively anneal to the 3' end of RNA, synthesising cDNA only from mRNA. Master mix was prepared based on the different components of the cDNA Reverse Transcription Kit.

Components of cDNA reverse transcription kit master mix:

Components	**Volumes (µl)**
g^{DNA} removal mix	2
Template RNA	4
RNase free water	9
Total	15

Table 1: Components for master mix of cDNA.

All the above components were mixed in PCR tubes for control (0mg/l) and 50mg/l NaF concentrations. Heat shock was done at 45ºC for 2 min using a thermal cycler and then immediately the tubes were snap chilled and spinned down.

Components	**Volumes (µl)**
Sample	15
RT Mix	4
RT enzyme	1
Total	20

Table 2: Master mix of cDNA.

Steps	**Temperature**	**Time**
Initialization	25ºc	3 mins
Denaturation	45ºc	90 mins
Annealing	85ºc	5mins
Extension	4ºc	Infinity

Table 3: Steps of PCR cycle.

Figure 8: Thermal cycler for PCR.

- **Real-Time qRT-PCR:**

Real-time Quantitative Reverse Transcription PCR was used to check the *fex* gene expression in NaF treated and non-treated plants from the prepared cDNA.

Forward and Reverse primers	**Annealing Temperature**
1F- AGCATTGTGCTGGCCATAGT	57.2ºC
1R- TGTATTTTCCGCTTTAGCCGTG	55.8ºC
6F-CGGAGACACCTCAAATGGCT	57.4ºC
8R- ATGCCCTTGCAATCTGACCA	57.3ºC

Table 4: Forward and reverse primers used for gene expression analysis.

Components	**volume**
SYBR Green	5 µl
Forward primer	0.8 µl
Reverse primer	0.8 µl
cDNA	1.0 µl
Nuclease free water	2.4 µl
Total	10 µl

Table 5: Reaction mix prepared for qRT-PCR.

Temperature	**Time**
95ºC	2 mins
95ºC	5 secs
37ºC	10 secs
65ºC-99ºC (Melting curve analysis)	

Table 6: Cycle conditions of qRT-PCR done upto 40 cycles.

Procedure:

The real time PCR amplification was done using SYBR Green detection chemistry. cDNA were run in triplicates on a 96 well reaction plates with the CFX Connect real time PCR (Bio-Rad, UK).

10 µl of the reaction mixture containing 5 µl SYBR Green Mix, 0.8 µl of the diluted primers and 1 µl of diluted cDNA as the template and 2.4 µl nuclease free sterile water was prepared and added in the 96 well reaction plates as shown in figure 9 and were run according to the amplification program shown in the Table 6 . The reference primers used, marked as gc in the plate, is of Eukaryotic elongation factor gene α, which is a constitutively expressed gene. It was used to see the expression in F affected rice plant.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Unk-1 1 0	Unk-1 1 0	Unk-1 1 0	Unk-2 1 50	Unk-2 1 50	Unk-2 1 50					
C		Unk-3 6/8 0	Unk-3 6/8 0	Unk-3 6/8 0	Unk-4 6/8 50	Unk-4 6/8 50	Unk-4 6/8 50					
D		Unk-5 gc 0	Unk-5 gc 0	Unk-5 gc 0	Unk-6 gc 50	Unk-6 gc 50	Unk-6 gc 50					
E												
F												
G												
H												

Figure 9: Plate set up for qRT-PCR.

Figure 10: BIO-RAD CFX Connect Real-time PCR system.

Chapter 4:

Results and discussion

Effect on Morphological Parameters

- **Germination percentage (GP):**

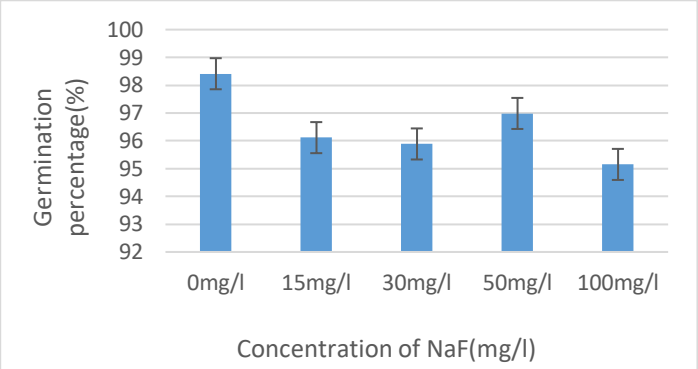
The germination percentage was calculated on the 3rd day of plating the seeds.

It was found that the seed germination in control was more compared to the seeds germinated in other concentrations of NaF. The percentage germination reduced at 15 mg/l NaF and the rate remained similar as concentrations of F increased.

Figure 11: Seeds grown on petri plates by giving different concentrations of NaF solutions i.e; 0 mg/l, 15 mg/l, 30 mg/l, 50 mg/l and 100 mg/l.

Concentration of NaF (mg/l)	Germination percentage (%)
0	98.41+/-1.19
15	96.11+/-1.57
30	95.89+/-0.90
50	96.98+/-1.13
100	95.15+/-0.81

Table 7: Germination percentage of the rice seeds at different concentrations of NaF.



Graph 1: Germination percentage of the rice seeds.

Significant reductions in percent seed germination in different crops have been reported at increasing F concentration (Sabal *et al*., 2006; Chandra *et al*., 2012). Our study corroborates with Gupta *et al*. (2009) who reported decreasing trend in germination with increasing concentration of NaF applied to rice.

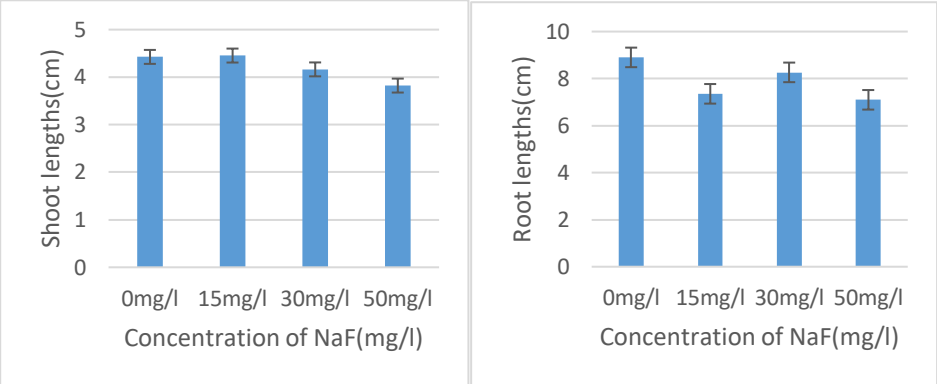
- **Shoot and root length:**

The shoot and root length of the rice plant was observed on 7th and 15th day.

As seen in the graph 2 that the shoot and root lengths of the rice seedlings are decreasing with increase in concentration of NaF, though the decrease was not significant. It can also be seen that the root length is greater as compared to the shoot length.

Concentration of NaF (mg/l)	**Shoot length (cm)**	**Root length (cm)**
0	4.43+/-0.38	8.90+/-0.90
15	4.45+/-0.40	7.35+/-2.13
30	4.16+/-0.38	8.26+/-0.82
50	3.82+/-0.27	7.10+/-0.79

Table 8: Shoot and root lengths of the rice seedlings subjected to different fluoride concentrations.



Graph 2: Bar graph showing the root and shoot lengths of the rice seedlings.

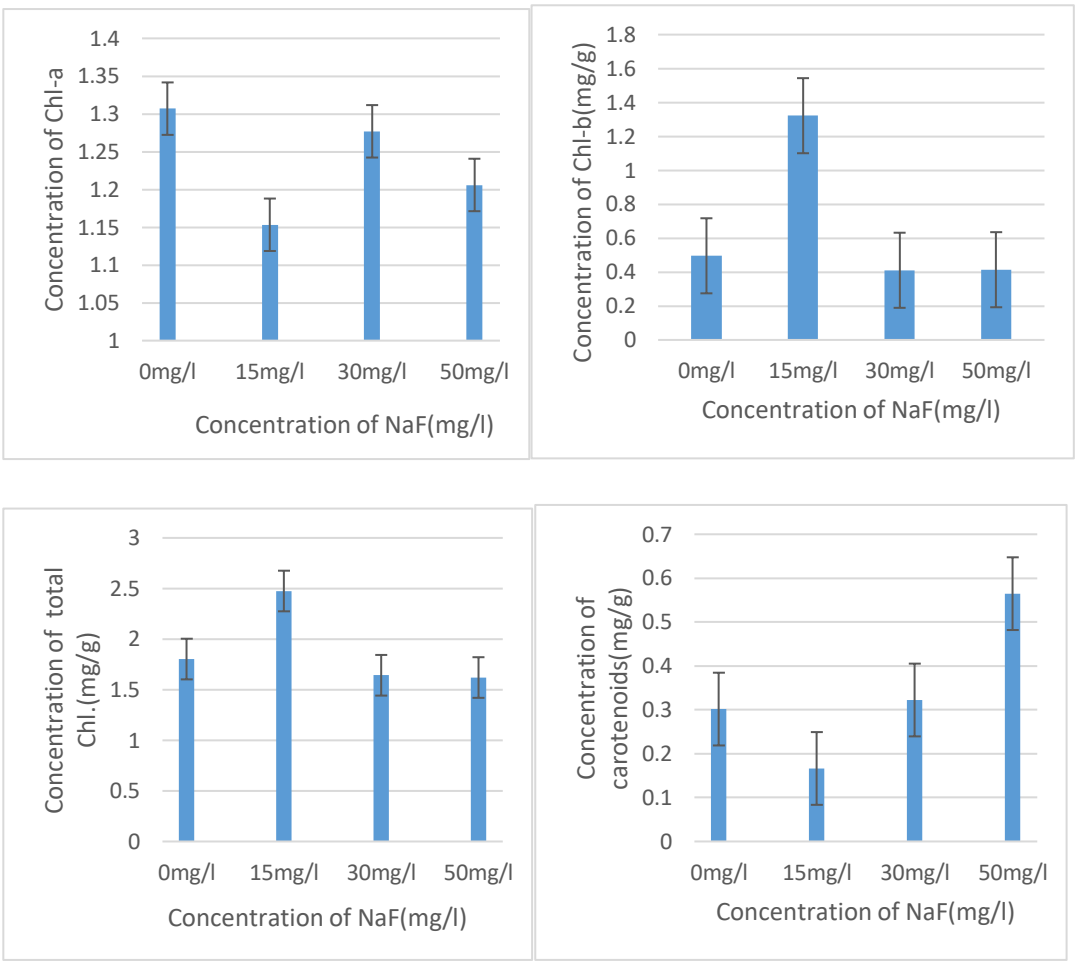
Gupta *et al.* (2009) observed a decrease in root and shoot length by 50 and 27 %, respectively by exposing rice seedlings to 30 mg/l NaF. However, in our case we did not observe any significant decrease in growth. A decrease in root and shoot lengths have also been observed by other authors (Sabal *et al*., 2006; Singh and Verma, 2013; Iram and Ti, 2016). We chose a different cultivar of rice and probably the phenotypic response of the plant to the toxicant varies in different cultivars.

Effect of fluoride on Biochemical constituents:

- **Pigment estimation:**

Concentration of NaF(mg/l	Chl-a(mg/g)	Chl-b(mg/g)	Total Chl (mg/g)	Carotenoids (mg/g)
0	1.31+/-0.28	0.50+/-0.11	1.80+/-0.34	0.30+/-0.09
15	1.15+/-0.27	1.32+/-1.06	2.48+/-1.22	0.17+/-0.10
30	1.28+/-0.33	0.41+/-0.08	1.64+/-0.43	0.32+/-0.08
50	1.21+/-0.37	0.41+/-0.21	1.62+/-0.57	0.56+/-0.21

Table 9: Effect of fluoride on the concentration of Chl-a, Chl-b, Total Chlorophyll and carotenoids.



Graph 3: Bar Graph Showing Concentrations of Chl a, Chl b, Total Chlorophyll and carotenoids.

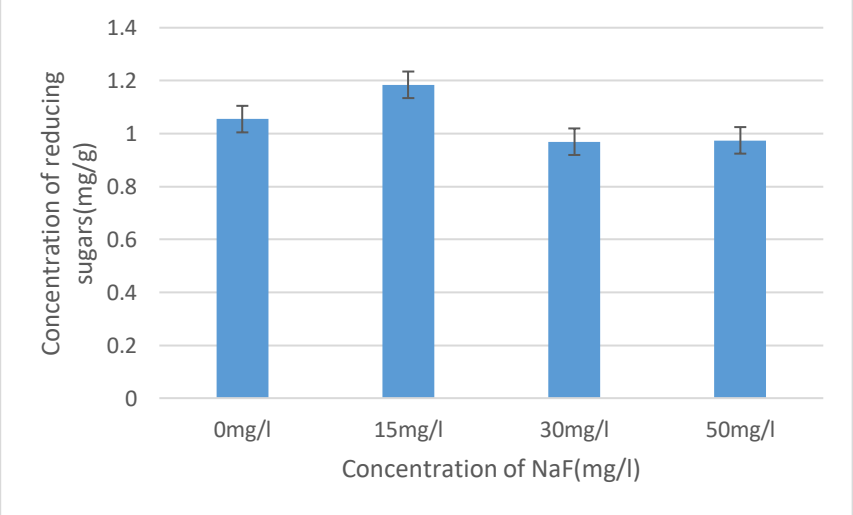
As seen from the table and the graph, chlorophyll content increased by 1.3 fold at 15 ppm NaF compared to control while it gradually decreased as the concentration of F was increased (30 and 50 ppm) and finally reached at a slightly lesser level relative to control. In contrast, carotenoid content decreased at 15 ppm F application and the gradually increased with increase in NaF. Various authors have indicated a decrease in chlorophyll and carotenoid levels with increased concentrations of NaF (Chandra *et al.*, 2012; Gupta *et al.*, 2013; Sing and Verma, 2013; Ram *et al.*, 2014). Our study though, reported a decrease in total chlorophyll content at higher concentration (50 ppm) in leaves, the carotenoid content increased by around 1.5 fold at that concentration of F, probably to compensate the negative effects of decreased chlorophyll. More studies are required to prove these results.

- **Estimation of Reducing sugar:**

Reducing sugar was estimated in the leaves of control and NaF-treated seedlings grown for 15 days. As seen in the graph, the concentration of reducing sugars in the leaves of paddy increased in 15 mg/l NaF as compared to control (0 mg/l) but then it decreased in 30 mg/l and 50 mg/l concentration.

Concentration of NaF(mg/l)	**Concentration of reducing sugars (mg/g)**
0	1.05+/-0.46
15	1.18+/-0.40
30	0.97+/-0.29
50	0.97+/-0.38

Table 10: Concentrations of reducing sugars (mg/g) in different NaF concentrations



Graph 4: Bar graph showing concentration of reducing sugars in leaves subjected to different concentration of NaF.

Contrasting results have been obtained by various authors on the content of total and reducing sugar as a response to F toxicity in different plant spp. According to Gupta *et al*. (2009) reducing sugar content initially decreases and then it gets increased with increase in NaF concentration. The increased level of reducing sugars may be due to its non conversion to non reducing sugars which might be a mechanism adopted by rice plants to minimise the effects of fluoride stress. Sodium fluoride (NaF) treatment

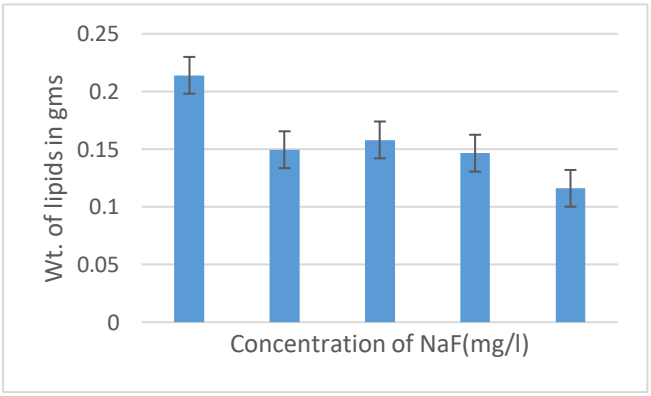
resulted in a significant enhancement of osmolytes such as proline and totalsoluble sugars content in *Vigna radiata* L (Gadi, 2012). Our results corroborated with Elloumi *et al*. (2005) decrease in reducing sugars and starch in the almond (*Amygdalis communis*) leaves with increasing F concentration in the nutrient solution. Since formation of reducing sugars such as glucose, fructose, and mannose in leaves is thought to be inhibited by F, the tendency of plants exposed to F to decrease the concentrations of such sugars in their leaves indicates the possible conversion of these sugars to non-reducing sugars, such as sucrose and raffinose or sugar alcohols (Elloumi *et al*., 2005). Under these conditions, increased levels of non-reducing sugars in tissues might be a mechanism adopted by plants to reduce F toxicity (Kim *et al*., 2003).

- **Estimation of Lipid content:**

The lipid content in the leaves was observed in the 15th day of plant growth. It was found that the content of the lipids decreased with increase in NaF concentraton.

Concentration of NaF(mg/l)	**Weight of Lipids (grams)**
0	0.214+/-0.02
15	0.150+/-0.001
30	0.158+/-0.002
50	0.147+/-0.004
100	0.116+/-0.01

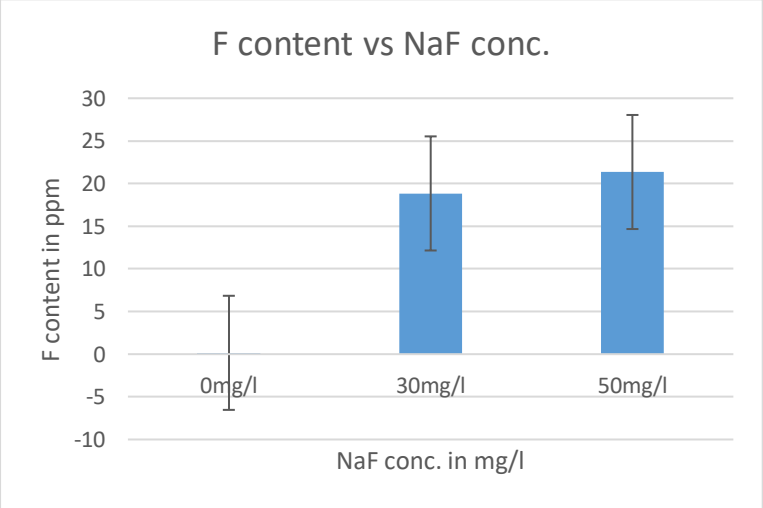
Table 11: Weights of lipid in rice leaves grown in different concentrations of NaF.



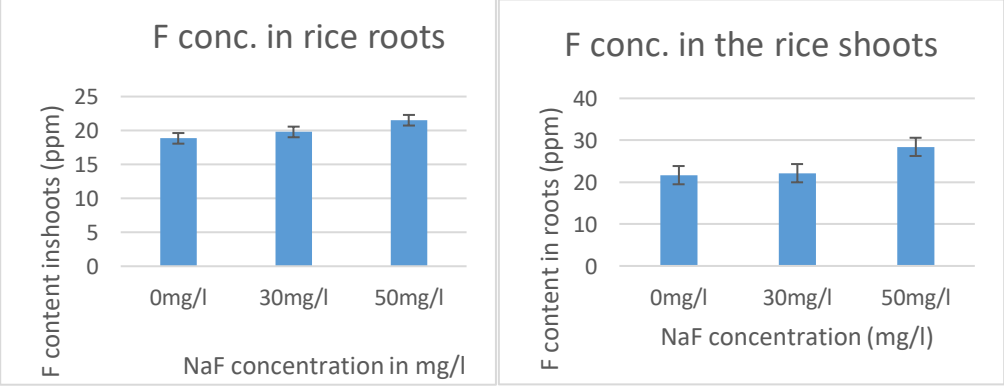
Graph 5: Weight of lipids in leaves subjected to different concentration of NaF.

F treatment can induce changes in lipid bilayer structure and alter the proportions of fatty acids (Zwiazek and Shay, 1988; Wang *et al.*, 2013). Lipids are esters of fatty acids and alcohols and comprise a large group of structurally distinct organic compounds that include fats, waxes, phospholipids, and glycolipids (Singh et al., 2002). As major components of membrane lipids, fatty acids play an important role in maintaining normal physiological cell function under environmental stress, including temperature, salt, chemicals, ions, pressure, and oxidative stress (Upchurch, 2008). The decreasing trends of lipid in response to increase in F indicates a negative effect on membrane integrity and normal physiological cell function on the plant. More study is required to establish the result.

- **Estimation of Fluoride accumulated:**



Graph 6: Fluoride content in different concentration of NaF solutions, i.e; 0 mg/l, 30 mg/l, 50 mg/l NaF prepared.



Graph 7: F accumulated in the shoots and roots of rice seedlings.

NaF accumulation is shown to increase as the rice plants are subjected to higher concentrations of NaF. It is also noted that more fluoride was accumulated in the shoot part of the plant. This may be due to the transportation of F from the roots to the shoots through te xylem and their deposition in the shoots.

- **Bioinformatic analysis of FEX Genes**

- **Determining rice FEX closest to Arabidopsis FEX**

FEX like protein from the model plant *Arabidopsis thaliana* is annotated as ORF At2g41705.1. This accession number was used to search the gene in TAIR (The Arabidopsis information resource) (https://www.arabidopsis.org/) and we got 3 splice variants of Arabidopsis FEX with accession numbers; AT2G41705.3, AT2G41705.4 and AT2G41705.2 (Fig 11).

Gene Model:

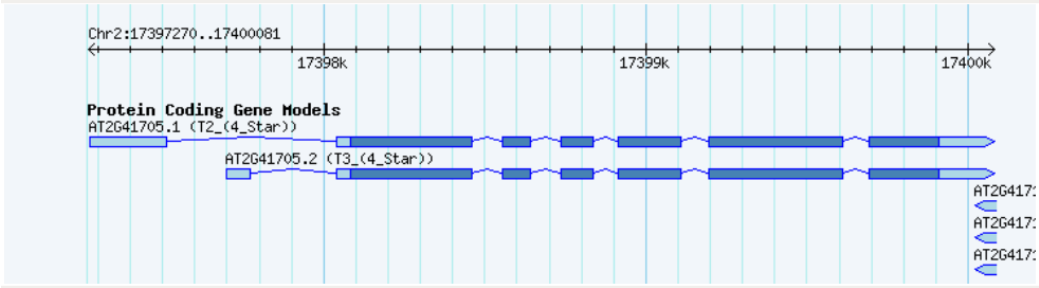


Figure 12 : Splice variants of Arabidopsis FEX genes. The Arabidopsis FEX like gene with accession number AT2G41705.1 was searched in TAIR. Three splice variants were obtained having accession number; AT2G41705.3, AT2G41705.4 and AT2G41705.2.

Protein sequence of *Arabidopsis thaliana* FEX:

```
>seq_90999 450 bases, 19BDFCBB checksum.
MDTGQSSIEPYQAKSFSRESSVASSLSLSRSLPHLIDNDVDSESVSEAGD
IGDRSLRRRHSAGRSSRLSADDFIEQGTHDTSRQEQDILHDLRAFNTASV
NKTLPEDITASPLPTKSLLSPEINNSGKEEERVLPKSLEYISCLIHLAVF
GIFGAITRYLLQKLFGPTGARVTSDGSILYLDLPSNMVGSFLMGWFGVVF
KADIARVSEFVAIGLSTGYLGSLTTFSGWNQKMLDLSADGQWVYAVLGFL
LGLFLTSYSIILGVETAKGFKWLLHRRASSEDKFHCLKVNTFQSHIVSLT
LMLLLLVALLTASSILLVKEFDKGTSEAQLWFGCLVAAPGVWLRWFLARL
NGRGLGKDSQNLRWVPFGTLIANVVAACVMAALATLKKSVNTRTCNTVAS
SIQFGLLGCLSTVSTFMAEFNAMRESDYPWRAYAYASFTIVVSFAIGTII
```

Mining of *Oryza* FEX genes using Arabidopsis FEX gene

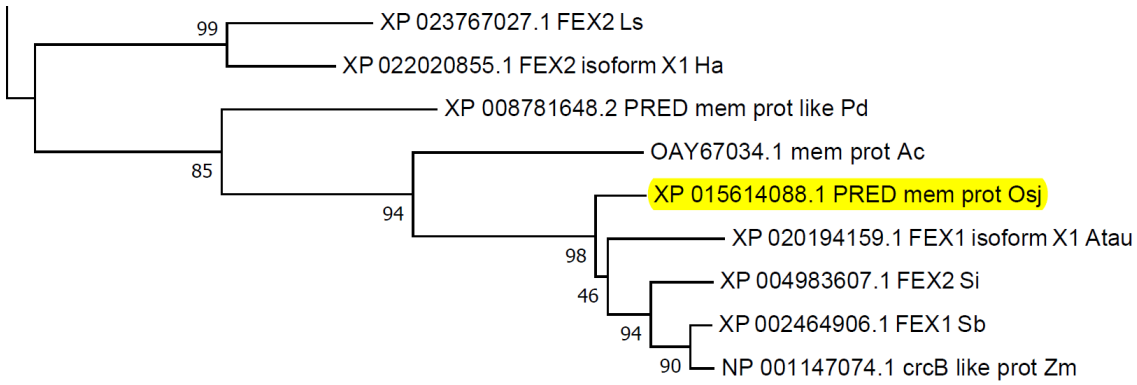
The protein sequence of Arabidopsis FEX protein (AT2G41705.1) was obtained from TAIR and saved as FASTA format. The FASTA protein sequence of Arabidopsis FEX was blasted in NCBI protein BLASTp(https://blast.ncbi.nlm.nih.gov), the algorithm parameter was changed to 500. Various sequences were obtained, out of which one was a predicted sequence for FEX of *Oryza sativa* Japonica group.

This predicted membrane protein sequence of *Oryza* was selected and converted into FASTA format. NCBI BLAST data was further verified by using the Arabidopsis FEX sequence and Blasting at Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/). Five crcB-like protein sequences were obtained which were found to be splice variants (Fig 12).

Gene Model

Figure 13: Gene model of the Splice variants of Oryza FEX proteins.

Phylogenetic Tree analysis:



From the phylogenetic tree analysis it has been predicted that the rice FEX is more evolved.

- **Prediction of subcellular localization of rice FEX gene:**

Subcellular localisation of rice and Arabidopsis FEX was predicted by using three different servers whose result has been shown in the below table:

Accession number	**Species**	**TargetP**	**WoLF PSORT**	**LocTree3**
AT2G41705	*Arabidopsis thaliana*	Chloroplast	Plastid	ER membrane
LOC_Os10g41749.2	*Oryza sativa*	Chloroplast	Plastid	ER membrane

It is predicted that the rice FEX protein may be localized either in ER membrane or in plastids. However, a dual localization may also be possible.

- **Prediction of Trans membrane domain in rice FEX.**

The presence of putative trans membrane domains in rice FEX was predicted by using three different web servers:
TMpred (https://embnet.vital-it.ch/software/TMPRED_form.html), CCTOP (http://cctop.enzim.ttk.mta.hu/),TMHMM (http://www.cbs.dtu.dk/services/TMHMM/)

It is predicted that probably there are 9 transmembrane helices present in rice FEX.

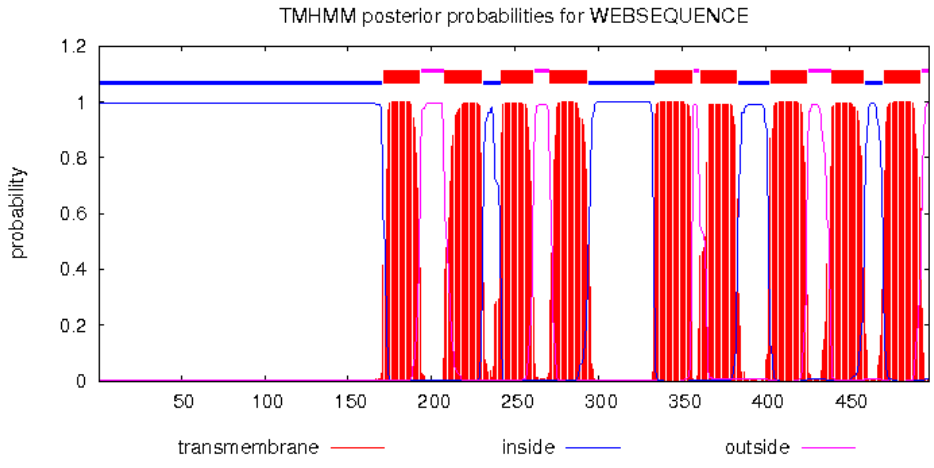


Figure 14: Prediction of Trans membrane helices in rice FEX.

Prediction of Rice FEX gene expression:

The rice FEX gene expressions subjected to various stresses was checked in Genevestigator. It was seen that the expression of FEX was downregulated due to the various stresses studied like, heat, cold, drought, pathogen attack and phosphorous. It was observed that the FEX expression was downregulated by 3.7 fold when subjected to phosphorous.

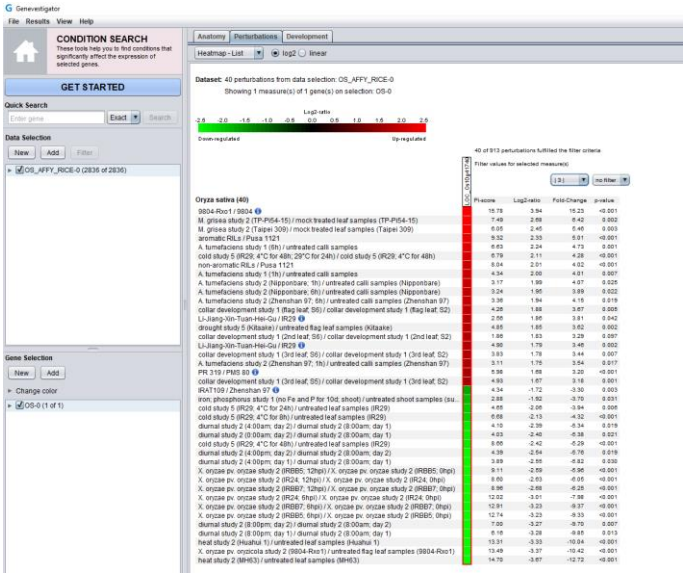
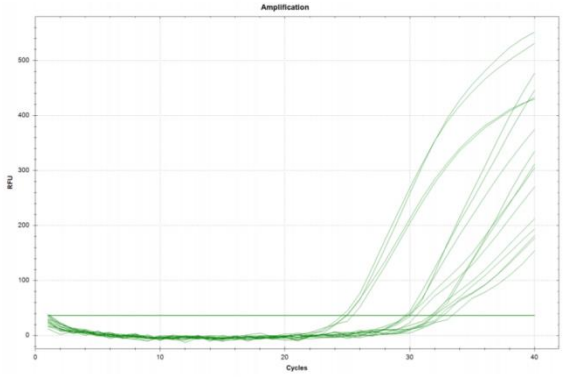


Figure 15: Rice FEX gene expression using Genevestigator.

- **Quantitative Real Time-PCR (qRT-PCR) analysis of rice *fex* ortholog**

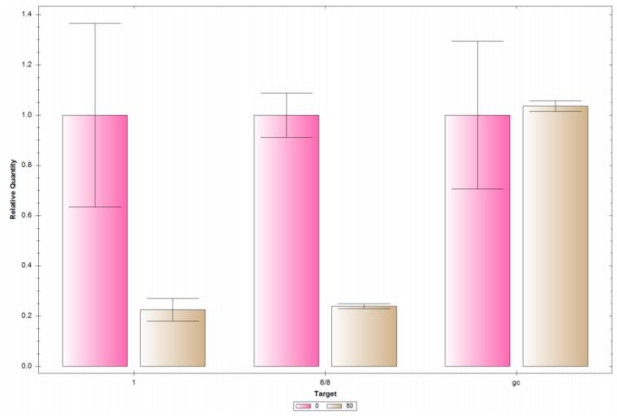
Real-time Quantitative Reverse Transcription PCR was used to check the *fex* (LOC_Os10g41749.2) gene expression in NaF treated and non-treated plants from the prepared cDNA.



Graph 8: Amplification of FEX and reference gene

Well	Fluor	Target	Content	Sample	Cq	Cq Mean	Cq Std. Dev
B02	SYBR	1	Unkn-1	0	29.85	30.68	0.914
B03	SYBR	1	Unkn-1	0	30.52	30.68	0.914
B04	SYBR	1	Unkn-1	0	31.66	30.68	0.914
B05	SYBR	1	Unkn-2	50	32.67	32.83	0.504
B06	SYBR	1	Unkn-2	50	32.43	32.83	0.504
B07	SYBR	1	Unkn-2	50	33.40	32.83	0.504
C02	SYBR	6/8	Unkn-3	0	30.06	29.83	0.220
C03	SYBR	6/8	Unkn-3	0	29.81	29.83	0.220
C04	SYBR	6/8	Unkn-3	0	29.62	29.83	0.220
C05	SYBR	6/8	Unkn-4	50	31.94	31.89	0.100
C06	SYBR	6/8	Unkn-4	50	31.78	31.89	0.100
C07	SYBR	6/8	Unkn-4	50	31.96	31.89	0.100
D02	SYBR	gc	Unkn-5	0	24.57	24.99	0.599
D03	SYBR	gc	Unkn-5	0	25.41	24.99	0.599
D04	SYBR	gc	Unkn-5	0	N/A	0.00	0.000
D05	SYBR	gc	Unkn-6	50	24.97	24.94	0.042
D06	SYBR	gc	Unkn-6	50	24.91	24.94	0.042
D07	SYBR	gc	Unkn-6	50	N/A	0.00	0.000

Table 12: Quantification data showing relative quantity (Cq)

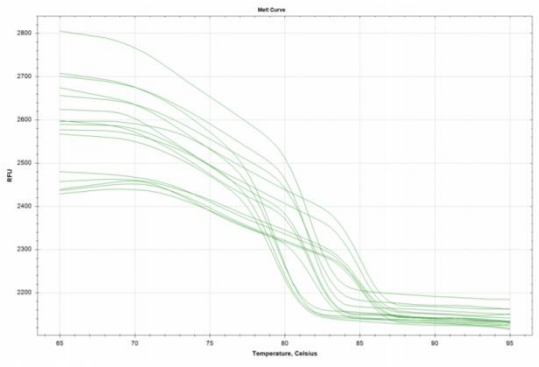


Graph 9: Bar graph showing FEX gene expression in NaF treated and non treated rice plant

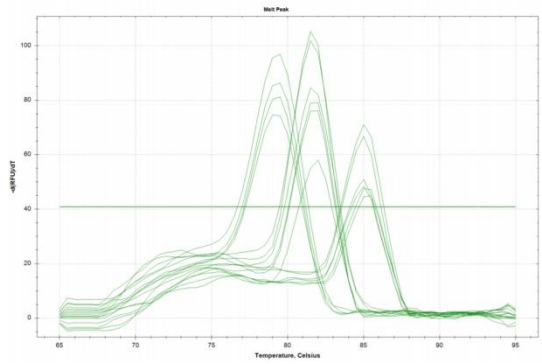
Gene Expression - Bar Chart Data

Target	Sample	Ctrl	Relative Quantity	Relative Quantity SEM	Corrected Relative Quantity SEM	Mean Cq	Cq SEM
1	0	*	1.00000	0.36561	0.36561	30.68	0.52746
1	50		0.22486	0.04539	0.04539	32.83	0.29120
6/8	0	*	1.00000	0.08819	0.08819	29.83	0.12724
6/8	50		0.23912	0.00959	0.00959	31.89	0.05783
gc	0	*	1.00000	0.29340	0.29340	24.99	0.42329
gc	50		1.03611	0.02124	0.02124	24.94	0.02957

Table 13: FEX gene expression in NaF treated and non treated rice plant



Graph 10: Melt curve of FEX and the reference gene



Graph 11: Melt peak of FEX and the reference gene

Well	Fluor	Target	Content	Sample	Melt Temp
B02	SYBR	1	Unkn-1	0	85.00
B03	SYBR	1	Unkn-1	0	85.00
B04	SYBR	1	Unkn-1	0	85.00
B05	SYBR	1	Unkn-2	50	85.00
B06	SYBR	1	Unkn-2	50	85.00
B07	SYBR	1	Unkn-2	50	85.50
C02	SYBR	6/8	Unkn-3	0	81.50
C03	SYBR	6/8	Unkn-3	0	81.50
C04	SYBR	6/8	Unkn-3	0	81.50
C05	SYBR	6/8	Unkn-4	50	82.00
C06	SYBR	6/8	Unkn-4	50	82.00
C07	SYBR	6/8	Unkn-4	50	82.00
D02	SYBR	gc	Unkn-5	0	79.50
D03	SYBR	gc	Unkn-5	0	79.50
D05	SYBR	gc	Unkn-6	50	79.50
D06	SYBR	gc	Unkn-6	50	79.00

Table 14: Melting temperatures

As seen from the graph, in response to high F (50 ppm) exposure, the transcript level of FEX gene was downregulated by around 5 fold relative to the control. In comparison, the reference transcript level was almost parallel in both control and F treated leaves. FEX (fluoride exporter) protein is essential for cell survival in the presence of high F concentrations. The protein is required for the rapid expulsion of cytoplasmic F, indicating that many eukaryotic species that carry FEX genes are likely to avoid F toxicity by purging cellular F (Li *et al*., 2013). In our study, downregulation of*FEX* expression in response to high F exposure corroborates with the *in silico* analysis of FEX gene expression to abiotic stresses and is indicative of sensitivity of this cultivar of rice towards F toxicity. More study is required to confirm this hypothesis.

Chapter 5:

Conclusion

Elevated concentration of fluoride in ground water is responsible for serious health problem in various parts of the world (Anbuvel *et al*., 2015). Two principal sources of fluoride intake by human in areas where fluoride concentration in ground water or surface water, and air are high are drinking water and edible crops. High fluoride concentrations are prevalent in ground waters from calcium-poor aquifers and in areas where fluoride-bearing minerals are common and have been reported from many countries including India (Fawell *et al.,* 2006). In India more than 66 million people are at the risk of developing fluoride toxicity due to ground water contamination. Plants absorb fluorine, both from the soil and air, as well as from falling atmospheric dust **(**Anbuvel et al., 2015). Rice (*Oryza sativa*) is a major dietary staple of nearly half the world's population .About 95% of this cereal is produced and consumed in South East Asian countries, including India. Thus, it becomes important to study how rice exposed to high F is affected and what is the mechanism of F accumulation in different cultivars of rice.

In this context, we studied the effect of high F on various morphological and biochemical parameters of rice cultivar CR Dhan 304, a commonly cultivated cultivar in Odisha and adjoining states. Additionally we carried out *in silico* expression analysis of rice ortholog of Arabidopsis *fex* gene and validated the result by qPCR expression analysis.

From our studies we found that

1) F affects the germination and growth of the rice plant. It showed decrease in the chlorophyll, reducing sugar and lipid content.
2) From the *in-silico* analysis we could predict 5 spliced variants of rice FEX proteins, and they are predicted to be present in the ER membrane and plastids and have 9 trans membrane helices.
3) *fex* gene expression analysis by qRT-PCR showed downregulation of *fex* gene when rice seedling was exposed to high concentration of NaF.

   The results suggest that exposure of high F to this variety of rice is detrimental and this variety may not be recommended for cultivation in high F areas.

Chapter 6:

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