ICAR Sponsored Winter School
on

Climate Change & its Impact on Photosynthesis and Productivity of Rice

5th-26th November 2018

PRACTICAL MANUAL

ICAR-National Rice Research Institute
Cuttack-753006, Odisha

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PRACTICAL MAUAL

Compiled by

Dr. M J Baig
Dr. P Swain
Dr. K Chakraborty

ICAR-National Rice Research Institute, Cuttack-753 006, Odisha

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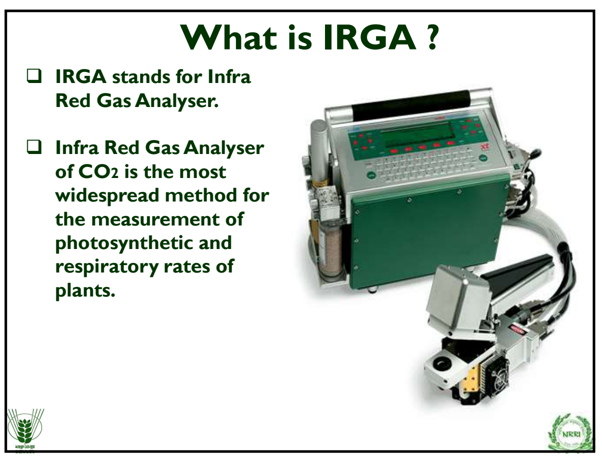
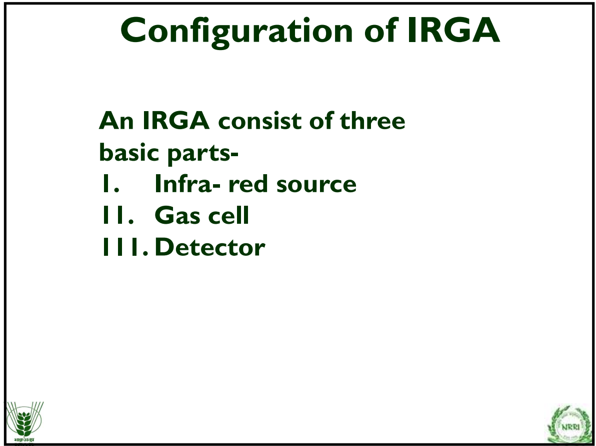
Gas Exchange Measurement by IRGA

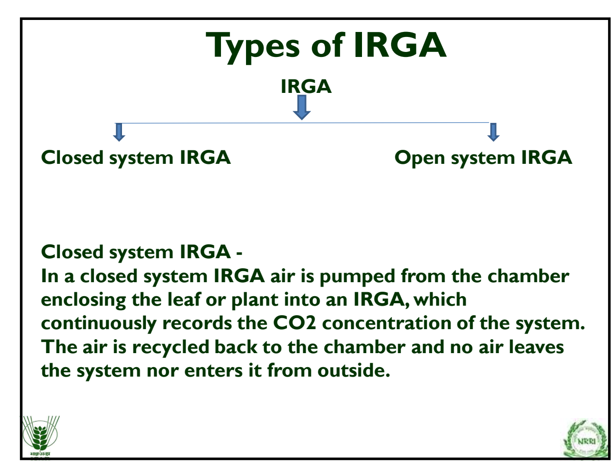
Koushik Chakraborty

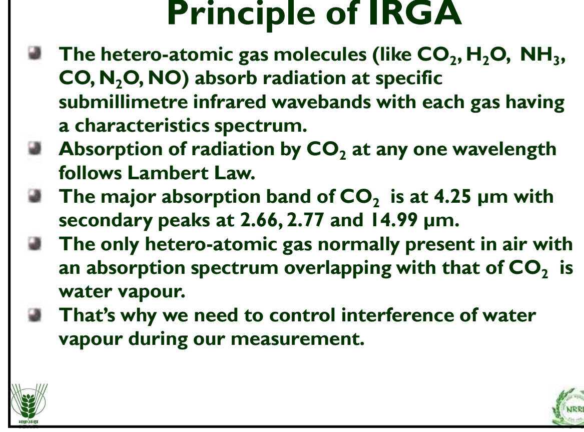
ICAR-National Rice Research Institute Cuttack, Odisha

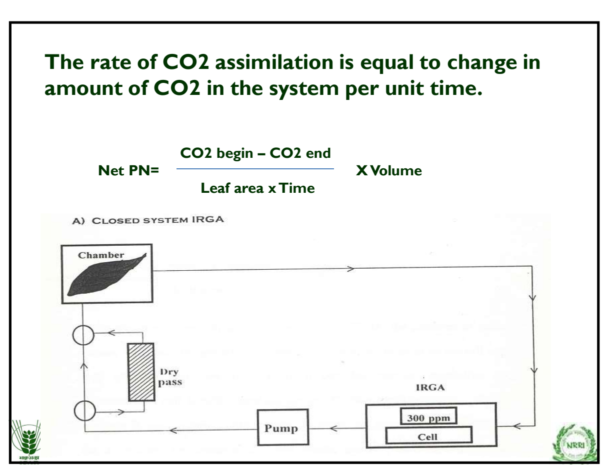

What is IRGA ?
IRGA stands for Infra Red Gas Analyser.
Infra Red Gas Analyser of CO_2 is the most widespread method for the measurement of photosynthetic and respiratory rates of plants.


Configuration of IRGA
An IRGA consist of three basic parts-
I. Infra- red source
II. Gas cell
III. Detector

Why should we use IRGA?
IRGA is safe, non-destructive, reliable, accurate, simple and less time consuming method to understand photosynthesis rate and stomatal conductance.


Types of IRGA
IRGA
Closed system IRGA
Open system IRGA
Closed system IRGA -
In a closed system IRGA air is pumped from the chamber enclosing the leaf or plant into an IRGA, which continuously records the CO2 concentration of the system. The air is recycled back to the chamber and no air leaves the system nor enters it from outside.

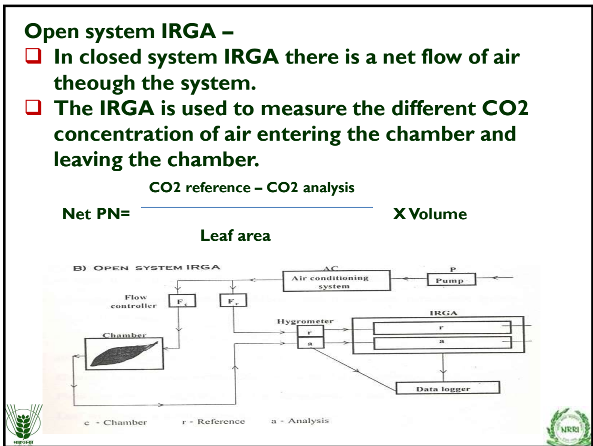

Principle of IRGA
The hetero-atomic gas molecules (like CO_2, H_2O, NH_3, CO, N_2O, NO) absorb radiation at specific submillimetre infrared wavebands with each gas having a characteristics spectrum.
Absorption of radiation by CO_2 at any one wavelength follows Lambert Law.
The major absorption band of CO_2 is at 4.25 µm with secondary peaks at 2.66, 2.77 and 14.99 µm.
The only hetero-atomic gas normally present in air with an absorption spectrum overlapping with that of CO_2 is water vapour.
That's why we need to control interference of water vapour during our measurement.


The rate of CO2 assimilation is equal to change in amount of CO2 in the system per unit time.
Net PN= $\frac{\text{CO2 begin} - \text{CO2 end}}{\text{Leaf area x Time}}$ X Volume
A) Closed system IRGA
Chamber
Dry pass
IRGA
300 ppm
Cell
Pump

Open system IRGA –

- In closed system IRGA there is a net flow of air theough the system.
- The IRGA is used to measure the different CO2 concentration of air entering the chamber and leaving the chamber.

$$\text{Net PN} = \frac{CO_2 \text{ reference} - CO_2 \text{ analysis}}{\text{Leaf area}} \times \text{Volume}$$



Working Procedure

- Firstly charge the batteries one day prior to record data using IRGA and load the charged batteries.
- Connect the CO2 tube to the inlet of the instrument in a proper way and very tightly otherwise it leaks(-ppm) on the display.
- The second tube was kept in empty thermocol box so that air can enter into the tube uniformly.
- Switch ON the instrument.

Advantages

Closed system IRGA

- Closed sysyems are the simplest configurations which enable quick measurements of PN of large samples of leaves.
- They require measurement of flow rate.

Open system IRGA

- Open system IRGA can be used to determine simultaneously the asscimilation rate(A) for a large number of chamber.
- They do not require the measurement of flow rate.

- Display shows-
  - A. welcome to loading open system.
  - B. Starting the net working.
  - C. It shows the fluorescence + WUE X ml- press Enter and the press Y for YES.
- Open the IRGA leaf chamber one time and close it and then select New Measurements and press F4.
- Again select Open Log Files and press F1.
  - Give the file name and press Enter.
  - Then give sub file name and again press Enter.
  - give date and press Enter.
- Next for CO2 matching –
  - Select Match(F5) and wait up to we get equal values of CO2 and sample CO2.
- To set the rows – m,n,c and 9 by pressing the alphabets m, n, c and 9 respectively and wait for 15-20 mins for warming the instrument.

Disadvantages

Closed system IRGA

- The measurements never represent truly steady stae responses; i.e. CO2 concentration changing, assimilation rate(A) cannot reach steady state.
- Recirculation of air within the system will result in a continuous rise in humidity.

Open system IRGA

- It cannot sense small differences in CO2 concentration between the two air stream.
- It requires an air conditioning system to control humidity and gas concentrations.

- Insert the leaf in IRGA chamber properly and
  Give the Dark pulse(F3) and then press 0 for getting 0 row.
  When F value is stable and df/dt value is <5, then select DOFoFm-(F3).
- After that select row 9 : : press Actinic ON(F4).
- Then select row no : 8 : press Define Actinic (F3)-
  It shows actinic definition then press Enter and type 1000 (PAR value 1000) then press Enter.
- Select 0 row as follow as described above.
- For taking Fluorescence value select O alphabet and note down the Fv/Fm value, then note down the IRGA readings (photosynthetic rate, transipiration, stomatal conductance).
- Before taking next reading Actinic is in OFF (F4).Dp as above for taking every next reading.

- After taking all the readings IRGA chambers must be in open conditions(loose the screw) and replace the fluorescence chamber foam(white foam).

Shutdown

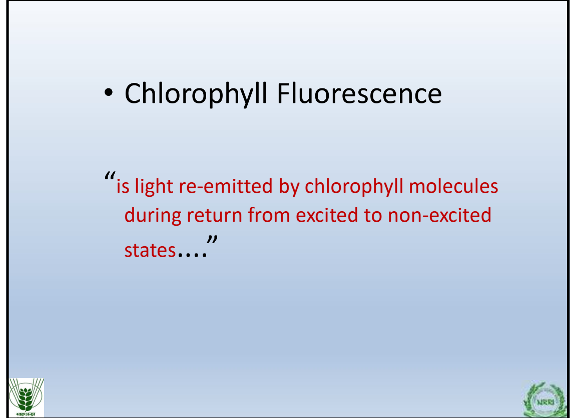
Actinic must be in OFF condition and press Escape button, then select Utility Menu- F5.

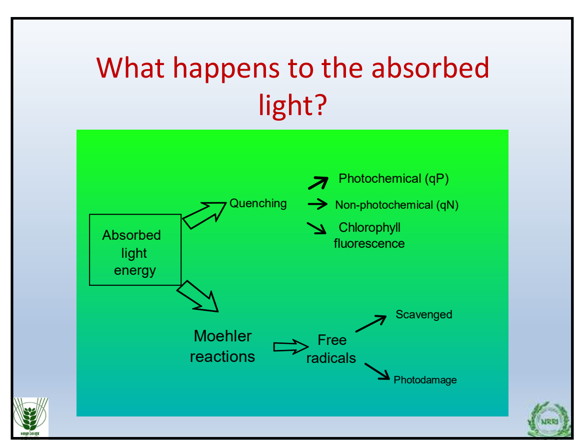
After that select Sleep and press Enter and Y for Yes.

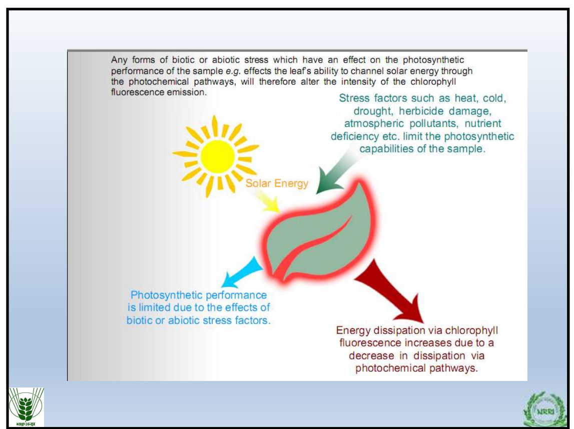
Switch off the system and disconnect the CO2 tube and keep the batterries for charging.

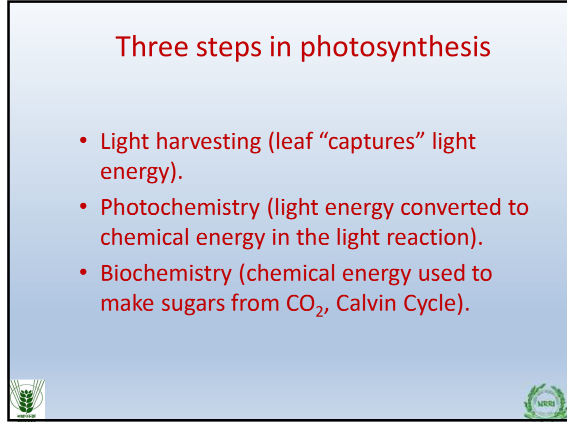
Chlorophyll fluorescence Measurement

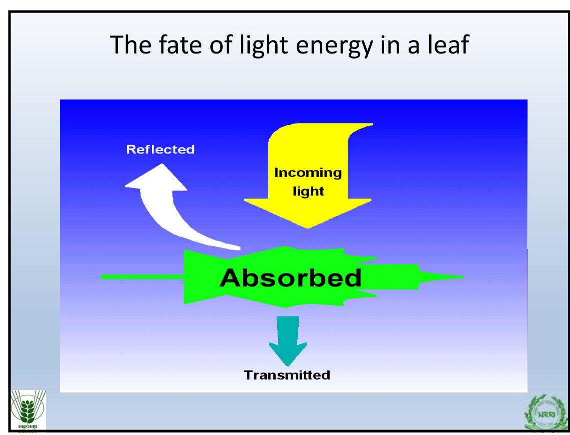
Dr Prashantkumar S. Hanjagi
Scientist (Plant Physiology)
ICAR-NRRI, Cuttack
Mb: 8308237819
Email: psh7160@gmail.com

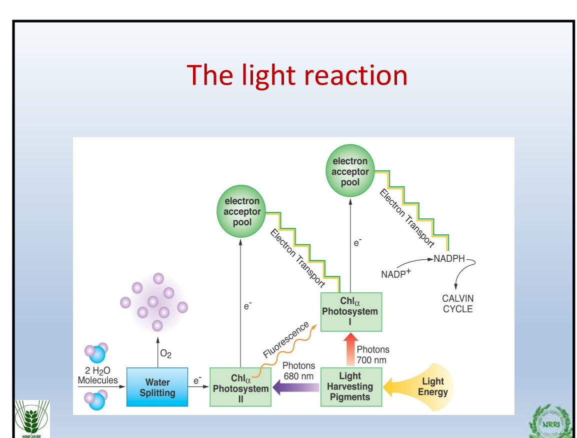

• Chlorophyll Fluorescence
"is light re-emitted by chlorophyll molecules during return from excited to non-excited states…."

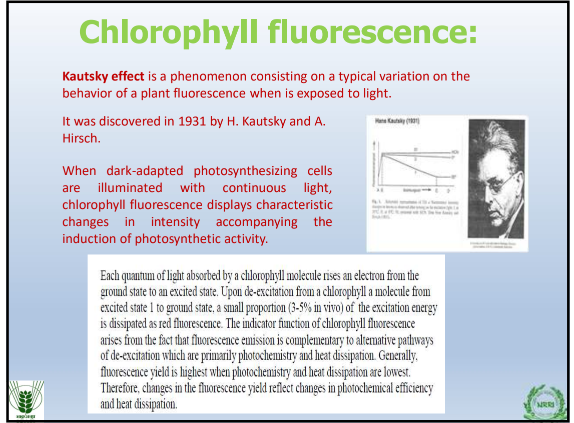

What happens to the absorbed light?
Absorbed light energy
Quenching
Photochemical (qP)
Non-photochemical (qN)
Chlorophyll fluorescence
Moehler reactions
Free radicals
Scavenged
Photodamage

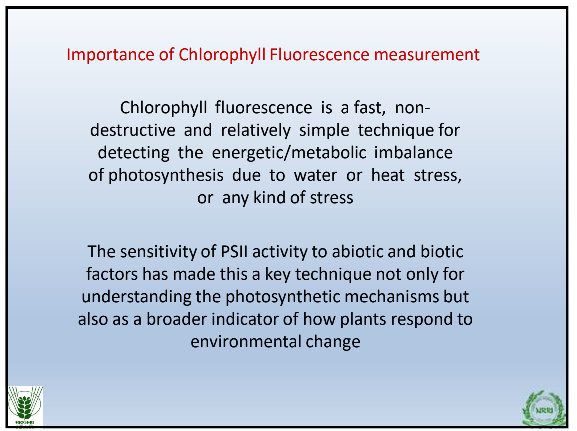

Any forms of biotic or abiotic stress which have an effect on the photosynthetic performance of the sample e.g. effects the leaf's ability to channel solar energy through the photochemical pathways, will therefore alter the intensity of the chlorophyll fluorescence emission.
Stress factors such as heat, cold, drought, herbicide damage, atmospheric pollutants, nutrient deficiency etc. limit the photosynthetic capabilities of the sample.
Solar Energy
Photosynthetic performance is limited due to the effects of biotic or abiotic stress factors.
Energy dissipation via chlorophyll fluorescence increases due to a decrease in dissipation via photochemical pathways.

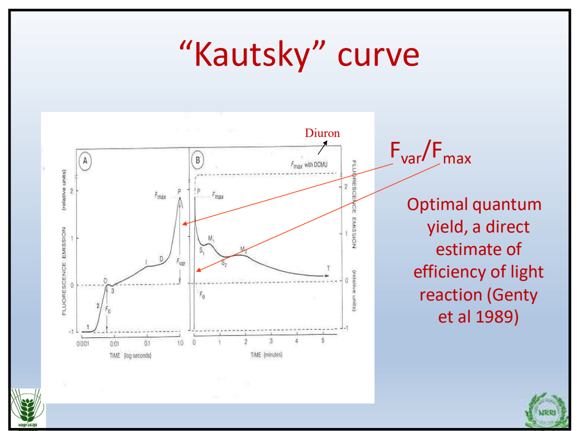

Three steps in photosynthesis
• Light harvesting (leaf "captures" light energy).
• Photochemistry (light energy converted to chemical energy in the light reaction).
• Biochemistry (chemical energy used to make sugars from CO_2, Calvin Cycle).

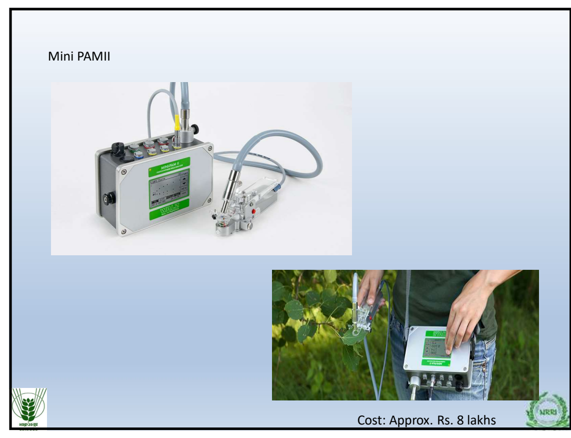

The fate of light energy in a leaf
Reflected
Incoming light
Absorbed
Transmitted

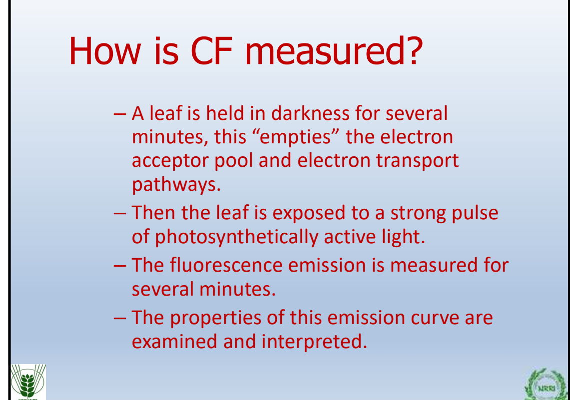

The light reaction
electron acceptor pool
electron acceptor pool
Electron Transport
Electron Transport
NADP+
NADPH
CALVIN CYCLE
Chl a Photosystem I
Photons 700 nm
Fluorescence
Photons 680 nm
2 H2O Molecules
O2
Water Splitting
Chl a Photosystem II
Light Harvesting Pigments
Light Energy

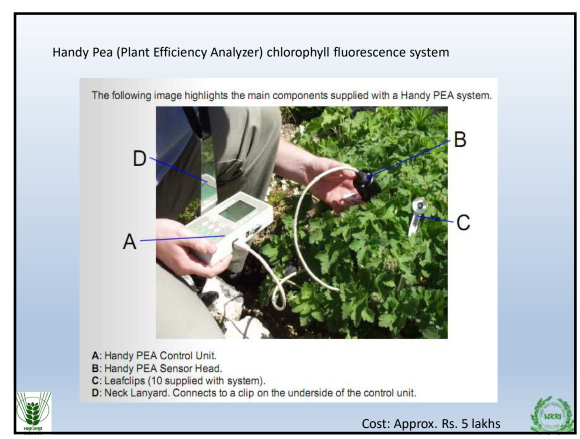

Chlorophyll fluorescence:
Kautsky effect is a phenomenon consisting on a typical variation on the behavior of a plant fluorescence when is exposed to light.
It was discovered in 1931 by H. Kautsky and A. Hirsch.
When dark-adapted photosynthesizing cells are illuminated with continuous light, chlorophyll fluorescence displays characteristic changes in intensity accompanying the induction of photosynthetic activity.
Each quantum of light absorbed by a chlorophyll molecule rises an electron from the ground state to an excited state. Upon de-excitation from a chlorophyll a molecule from excited state 1 to ground state, a small proportion (3-5% in vivo) of the excitation energy is dissipated as red fluorescence. The indicator function of chlorophyll fluorescence arises from the fact that fluorescence emission is complementary to alternative pathways of de-excitation which are primarily photochemistry and heat dissipation. Generally, fluorescence yield is highest when photochemistry and heat dissipation are lowest. Therefore, changes in the fluorescence yield reflect changes in photochemical efficiency and heat dissipation.


Importance of Chlorophyll Fluorescence measurement
Chlorophyll fluorescence is a fast, non-destructive and relatively simple technique for detecting the energetic/metabolic imbalance of photosynthesis due to water or heat stress, or any kind of stress
The sensitivity of PSII activity to abiotic and biotic factors has made this a key technique not only for understanding the photosynthetic mechanisms but also as a broader indicator of how plants respond to environmental change


"Kautsky" curve
Diuron
Fvar/Fmax
Optimal quantum yield, a direct estimate of efficiency of light reaction (Genty et al 1989)


Mini PAMII
Cost: Approx. Rs. 8 lakhs

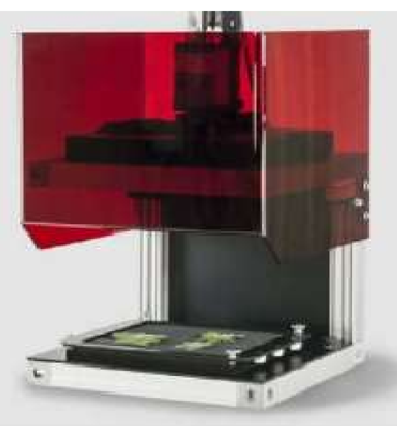

How is CF measured?
– A leaf is held in darkness for several minutes, this "empties" the electron acceptor pool and electron transport pathways.
– Then the leaf is exposed to a strong pulse of photosynthetically active light.
– The fluorescence emission is measured for several minutes.
– The properties of this emission curve are examined and interpreted.


Handy Pea (Plant Efficiency Analyzer) chlorophyll fluorescence system
The following image highlights the main components supplied with a Handy PEA system.
D
B
C
A
A: Handy PEA Control Unit.
B: Handy PEA Sensor Head.
C: Leafclips (10 supplied with system).
D: Neck Lanyard. Connects to a clip on the underside of the control unit.
Cost: Approx. Rs. 5 lakhs

LICOR-6400-40 Leaf Chamber Fluorometer
Cost Approx. Rs 38 Lakhs

Chlorophyll Image Imaging

IMAGING–PAM MAXI VERSION



Dr Koushik Chakraborty (Scientist)
Plant Physiology and Biochemistry.
National Rice Research Institute

Introduction

- Chlorophyll fluorescence is a popular technique used in plant physiology to measure Photosystem II (PS-II) activity.
- Excited chlorophyll dissipates the absorbed light energy by driving photosynthesis (photochemical energy conversion), as heat in non-photochemical quenching or by emission as fluorescence radiation.

- To understand Chlorophyll fluorescence completely we had to know about-

1. What is chlorophyll fluorescence
2. Principles of Chlorophyll fluorescence analysis.
3. Commonly used fluorescence parameters.

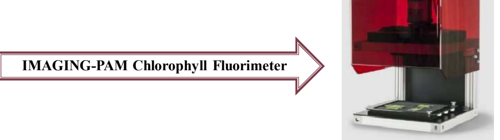
Introduction

- The IMAGING-PAM Chlorophyll Fluorometer is specialized for the study of two-dimensional heterogeneities of photosynthetic activity.
- MAXI-, MINI- and MICROSCOPY are the three versions have been issued that are operated with the same Multi Control Unit IMAG-CG. Like all PAM fluorometers, the Imaging-PAM applies pulse-amplitude-modulated measuring light for assessment of chlorophyll fluorescence yield.

What is chlorophyll fluorescence

- Chlorophyll within leaf present as pigment protein complexes in PS-I ,PS-II and within light harvesting complexes(LHC).
- Light energy absorbed by the chlorophyll molecules can

1. be drive photosynthesis.
2. be re-emitted as heat.
3. be re-emitted as light (fluorescence).



The basic measuring system of the MAXI- Imaging PAM consists of five units

1. Control Unit IMAG-CG with battery charger
2. LED array illumunation unit IMAG-MAX/L(Blue)
3. CCD Camera IMAG-K7
4. Mounting stand with eye protection
5. PC with Imaging Win software

What is chlorophyll fluorescence

- The 3 processes presents in plant and works in competitive manner. Therefore, yield of chlorophyll fluorescence emission gives us important information about photosynthesis (Photochemistry), heat dissipation and information about photosynthesis helps to understand productivity of the plant.

Principles of Chlorophyll Fluorescence Analysis

- When a light sufficient to drive photosynthesis is applied a dark adapted leaf , there is transient rise in the level chlorophyll fluorescence. That is usually result of reduction of electrons in the thylakoid membrane.
- The chlorophyll in PS-II (P-680) ejects an electron derived from water splitting to the electron acceptor (Quinone) via initial acceptor pheophytin.

Commonly used fluorescence parameters

- Fo= Minimal fluorescence of a dark adapted sample when all reaction centers are open.
- Fm= Maximum fluorescence of a dark adapted sample when high intensity light has been applied .All reaction centers of the PS-II are closed.
- Fo'= Minimal fluorescence of a light adapted sample when all reaction centers are open.

Principles of Chlorophyll Fluorescence Analysis

- Quinone is not able to accept another electron from P680 until it was passed its electron next carrier (Quinone). This time reaction centers is considered to be closed and closure will inevitably cause a decline in quantum efficiency of PS-II, means initial rise in fluorescence after application of light and fluorescence signal then declines over a period of minutes, which is termed as Quenching. Quenching can arise from a combination of processes-
- (A) Light activation of photosynthesis –Here key enzymes of calvin cycle requires activation in order to achieve full activity.

Commonly used fluorescence parameters

- Fm'= Maximum fluorescence of a light adapted sample when all reaction centers are closed.
- F'= Steady state level of fluorescence in the light adapted sample.
- Fv= Variable fluorescence

  Fv= Fm-Fo

Principles of Chlorophyll Fluorescence Analysis

- (B) On illumination, there is a rapid increase in the rate constant for heat dissipation of chlorophyll excitation energy, measured by a parameter NPQ(Non photochemical Quenching) This is a photo protective process that removes excess excitation energy within chlorophyll and prevents formation of damaging free radicals

Commonly used fluorescence parameters

- Fv/Fm is the ratio of variable and maximum fluorescence for dark adapted samples calculated as Fm-Fo/Fm. This was maximum quantam efficiency of PS-II.
- Fv'/Fm' is the ratio of variable and maximum fluorescence for light adapted samples calculated as Fm'-Fo'/Fm'. This was maximum efficiency of PS-II if all centers were open.

Commonly used fluorescence parameters

- If we have both dark adapted and light adapted measurements we can measure 3 key parameters-
- The operating efficiency of PS-II photochemistry =Fq'/Fm',(Fm'-F'/Fm') often called YPS(II) and used as a most common parameter for light adapted samples.

Commonly used fluorescence parameters

- Coefficient of nonphotochemical quenching(qN) is defined by equation

  $qN= (Fm-Fm')/(Fm-Fo')$.
- Quantum yield of regulated energy dissipation Y(NPQ) can be calculated by

  $Y(NPQ)=1-Y(II)-1/[NPQ+1+qL(Fm/Fo-1)]$.

Commonly used fluorescence parameters

- Fq'/Fm' can broken further-
- The level of photochemical quenching of PS(II) gives an indication of the proportion of reaction centers that are open, often called qP

  $qP= (Fm'-F')/(Fm'-Fo')$
- Another one is PS-II maximum efficiency a parameter that describes the maximum operating efficiency inn the light adapted state can be calculated with Fv'/Fm'.

Commonly used fluorescence parameters

- Quantum yield of non regulated energy dissipation Y(NO) can be calculated by

  $Y(NO)=1/[NPQ+1+qL(Fm/Fo-1)]$.

Commonly used fluorescence parameters

The parameter qL considered more accurate indicator of PS-II redox state and estimates the fraction of reaction centers that are open.

$qL= (Fq'/Fv')/(Fo'/F')$.

Nonphotochemical quenching(NPQ) estimates the rate constant for heat loss for PS-II.

$NPQ= Fm-Fm'/Fm'$.

Working procedure

- Keep the leaves dark adapted for at least 20-30 minutes before sampling.
- Bring the dark adapted leaves to the laboratory wrapped in wet clothes to keep leaves turgid and away from light source.
- Take at least 5 random lesf samples (leaf piece of 5cm) for imaging.
- Switch on the instrument and the PC connected to it.

Working procedure

- Switch off the lights in the surrounding to avoid unnecessary light exposure to leaves.
- Place the leaf samples carefully in the stage inside the metal filaments giving enough spacing between two samples.
- Click on the desktop icon ‘ ImageWinGigE’ to start the programme.
- Select ‘MAXI’ unit from the pop-up window.

Advantages of Chlorophyll Fluorescence Imaging.

- It’s rapid and cost effective process for understanding Photosystem -II activity.
- Chlorophyll fluorescence technique helps us to understand the fundamental mechanisms of photosynthesis, behavior of plants under different environmental conditions and genetic variation etc.

Working procedure

- Carefully position the areaof measurement in different points on the leaf samples by using Add/Delete button on right hand panel
- Once the position is finalized, click on the ‘FoFm’ button to give first pulse of light.
- After that, click on the ‘SAT-Pulse’ button to give saturating light.
- Now click on different image types to get desired images.

Disadvantages of Chlorophyll Fluorescence Imaging.

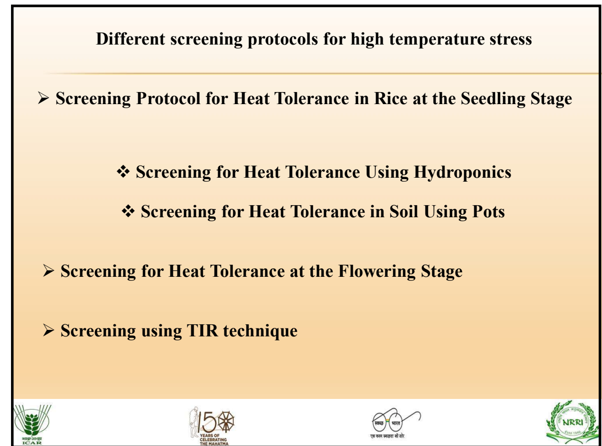
- Choice of parameter is important as sometimes wrong choice of ChlF trait may not yield clear results.
- Fluorescence measurements do not distinguish populations or progenies of plant species when growing under a non-stressful environment.

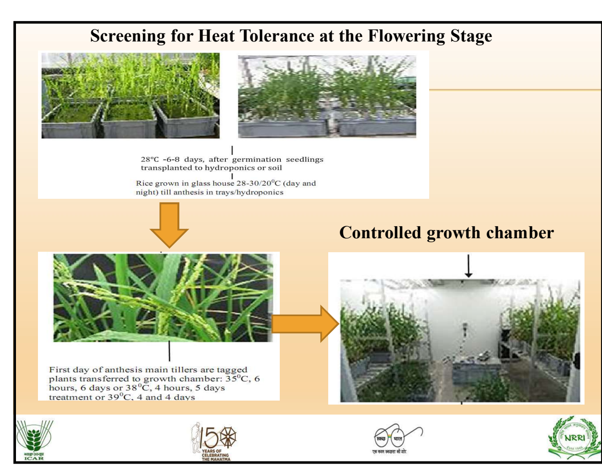
Working procedure

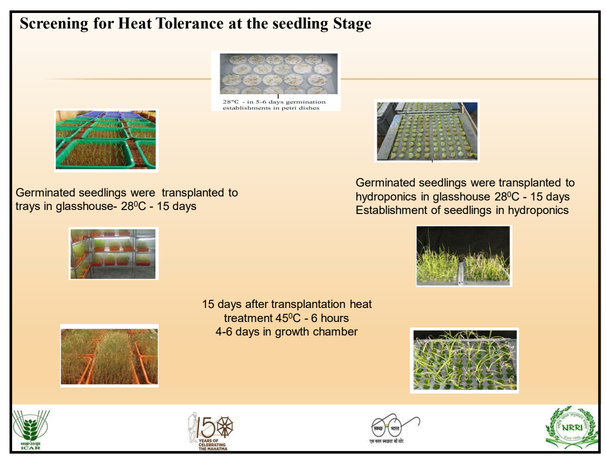
- Save the images(as .PIM file) by clicking on the ‘Export’ button for future use
- For the light curve analysis go to ‘LightCurve’ menu and select the ranges on light intensities.
- Go to ‘Report’ menu for the data of respective parameters and export them as .XLSX files.
- Close the programme and switch off the PC and instrument after use

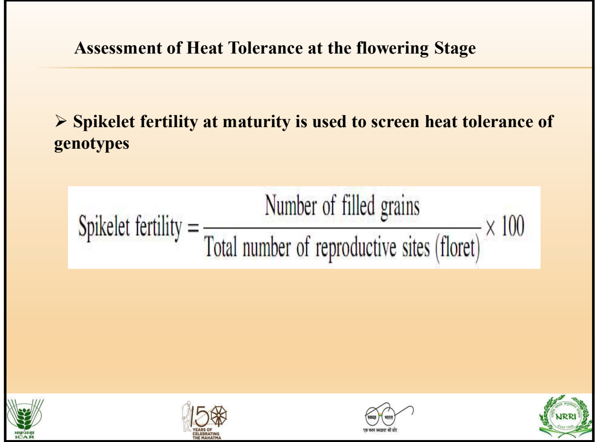
Screening methodologies for identifying rice genotypes for tolerance to high temperature

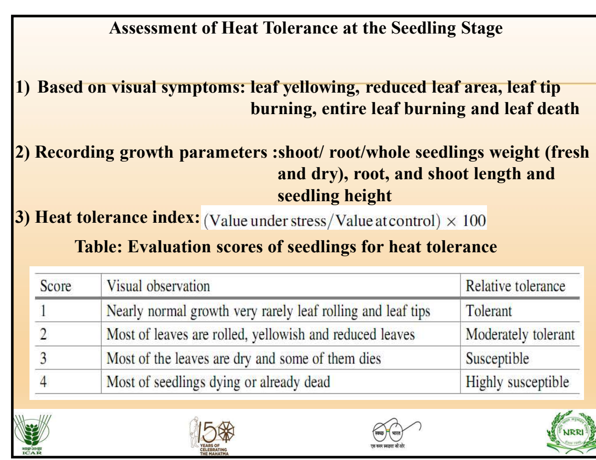
Dr. Prashantkumar. S.Hanjagi
Scientist
CPBD
ICAR-NRRI



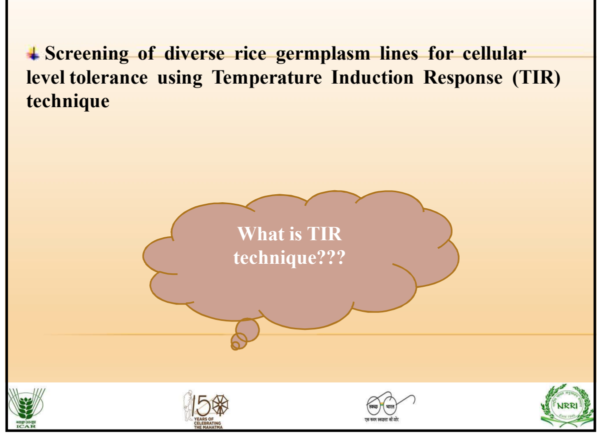


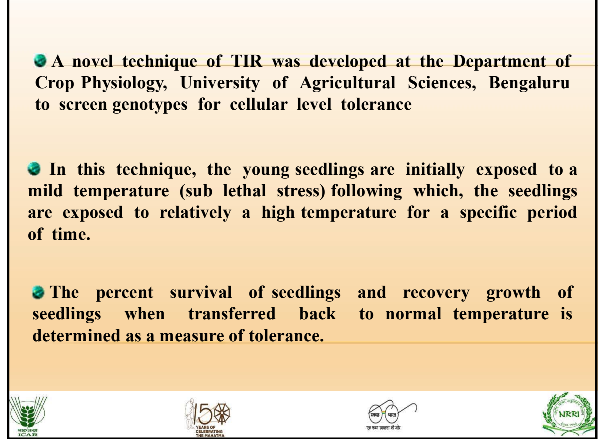


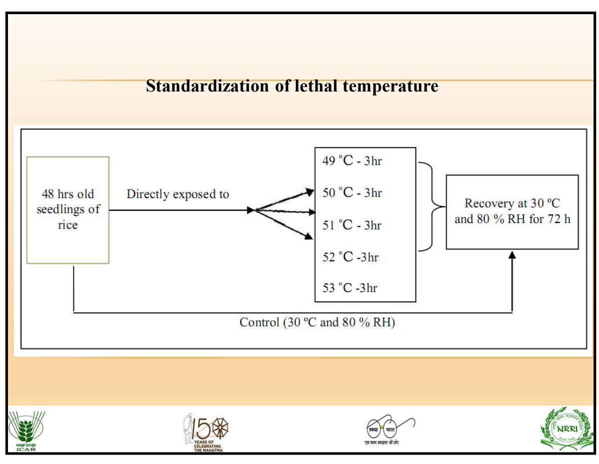


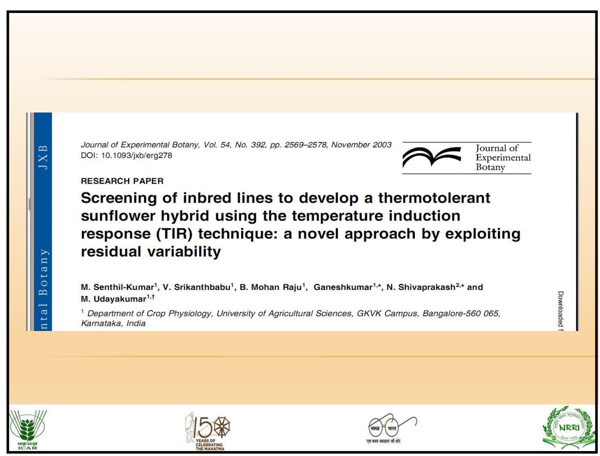


Score	Visual observation	Relative tolerance
1	Nearly normal growth very rarely leaf rolling and leaf tips	Tolerant
2	Most of leaves are rolled, yellowish and reduced leaves	Moderately tolerant
3	Most of the leaves are dry and some of them dies	Susceptible
4	Most of seedlings dying or already dead	Highly susceptible







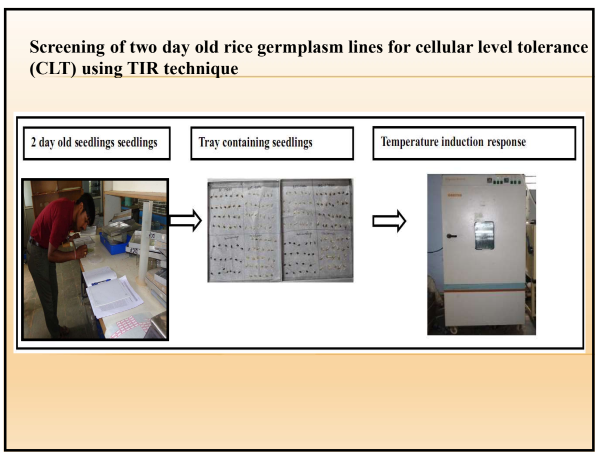

Journal of Experimental Botany, Vol. 54, No. 392, pp. 2569–2578, November 2003
DOI: 10.1093/jxb/erg278

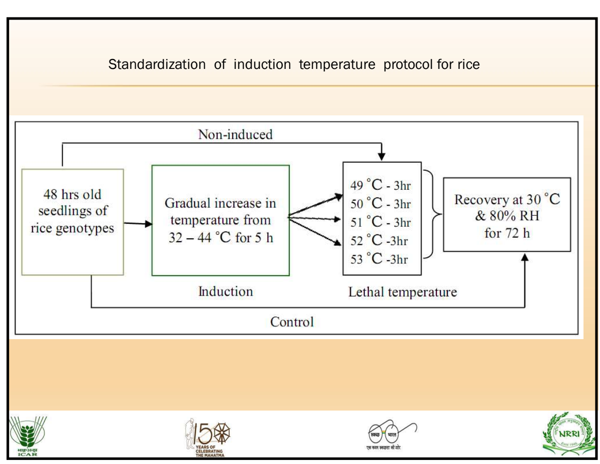
RESEARCH PAPER

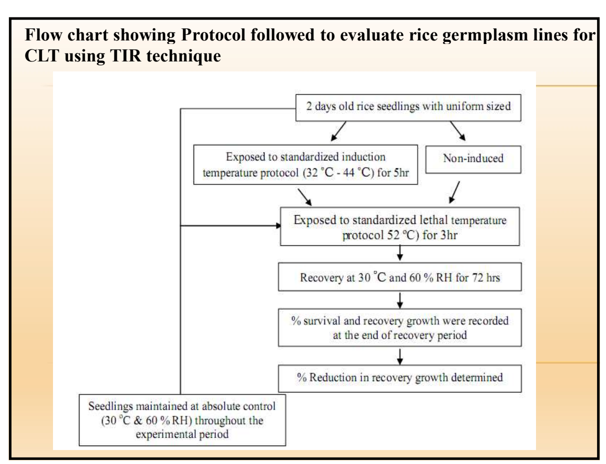
Screening of inbred lines to develop a thermotolerant sunflower hybrid using the temperature induction response (TIR) technique: a novel approach by exploiting residual variability

M. Senthil-Kumar[1], V. Srikanthbabu[1], B. Mohan Raju[1], Ganeshkumar[1,*], N. Shivaprakash[2,*] and M. Udayakumar[1,†]

[1] Department of Crop Physiology, University of Agricultural Sciences, GKVK Campus, Bangalore-560 065, Karnataka, India







➢ Calculation of survival percentage and recovery growth

$$\text{Survival (\%)} = \frac{Ns}{N} \times 100$$

Where,

Ns = Number of seedlings survived after recovery period

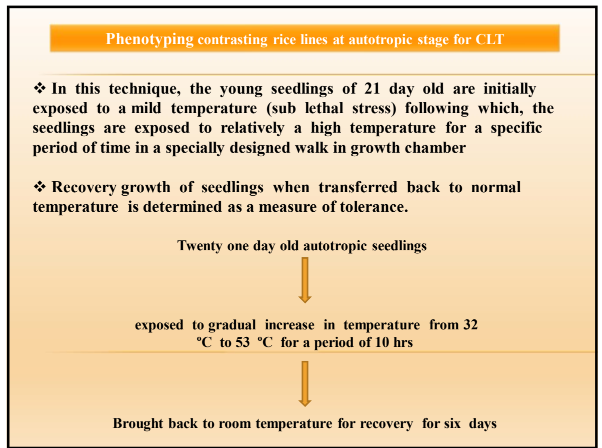
N = Number of seedlings taken

▪Recovery growth = Final growth of root & shoot – Initial growth of root & shoot

RRG (%) = {AGC-RGI}/AGC

Where,

AGC= Absolute growth of control seedlings
RGI= Recovery Growth of Induced seedlings



Identification of contrasting rice germplasm lines differing in CLT

❖ Genotypes contrasting for CLT will be selected based on the recovery growth and per cent RRG

❖ Accordingly, any genotype to be considered as tolerant ones, it should not only survive under stress, but also put on more recovery growth

❖ The recovery growth and per cent RRG values will be transformed to generate standardized normal distribution values (Z values) and plotted against each other

Screening of twenty one day old rice contrasting lines for cellular level tolerance (CLT) at whole plant level

The Z - values for different parameters were determined as follows

$$\frac{X - Xi}{SD}$$

Where,
X: Overall mean of all the genotypes for a character.
Xi: Mean of individual genotype.
SD: Standard deviation.

+ve
1st quadrat (Low CLT types)
2nd quadrat
% RRG 0
4th quadrat
3rd quadrat (High CLT types)
-ve
-ve 0 +ve
Recovery growth

N
R
R
I

Screening Techniques to Identify Rice Genotypes Tolerant to Submergence & Salinity

Koushik Chakraborty, Ph.D.
Scientist (Sr. Scale), Plant Physiology
Division of Crop Physiology & Biochemistry
ICAR-National Rice Research Institute

Introduction

•Soil salinity is the second most major and increasing problem limiting rice growth and leads to huge yield losses every year, after drought.

•Salt tolerance is generally a sustained growth of the plant in the soil environment impregnated with NaCl or other salt combinations.

Nutrient solution

Micronutrient solution

a. MagnousChloride(0.074gm)
b. Ammonium Molebdate (0.035gm)
c. Zinc Sulphate(0.934gm)
d. Boric Acid (0.934gm)
e. Cupric Sulphate (0.031gm)
f. Ferric Chloride (7.7gm)
g. Citric Acid (11.9gm)

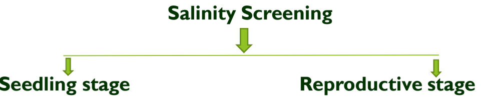
Macronutrient solution

a. Ammonium Nitrate (91.4gm)
b. Sodium Phosphate(35.6gm)
c. Potassium Sulphate(71.4gm)
d. Calcium Chloride(117.5gm)
e. Magnesium Sulphate (324gm).

Screening of Salinity

Several studies indicated that rice is tolerant during germination, becomes very sensitive during early seedling stage (2–3 leaf stage), gains tolerance during vegetative growth stage, again becomes sensitive during pollination and fertilization and then becomes increasingly more tolerant at maturity or reproductive stage.

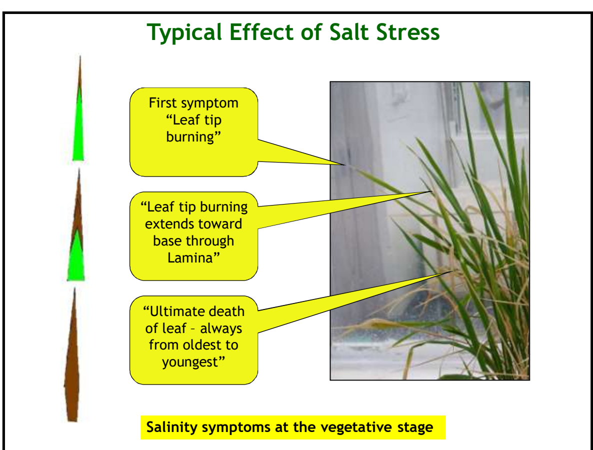
Salinity Screening



Seedling stage

Reproductive stage

Typical Effect of Salt Stress



Salinity symptoms at the vegetative stage

Screening at seedling stage

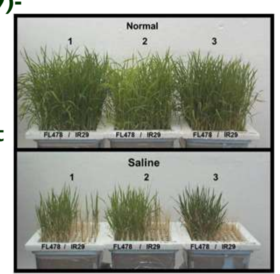
•Rice is very sensitive to salinity at seedling stage.

•The screening techniques developed is based on the ability of seedlings to grow in salinized nutrient solution.

Scoring of plants

Scoring of salinity affected plant can be done using these numbers according to morphological appearance with reference to highly susceptible (IR-29)-

- 9- Highly susceptible
- 7- Susceptible
- 5- Moderately tolerant
- 3- Tolerant
- 1- Highly tolerant



Working Procedure

- The seeds of the variety to be screened must be pre-heated in hot air oven for 3-5 days at 50°C to break seed dormancy.
- These sterile seeds were then placed in petri-dishes with moistened filter papers and incubated at 30°C for 48 hours to germinate.
- Two seeds of the each genotype with a tolerant and susceptible variety, were sowed in per hole on the Styrofoam seedling float in such a manner, so that the radical was inserted through the nylon mesh.

Screening at reproductive stage

- To determine the salinity tolerance at reproductive stage is quite difficult.
- Salinity stress at reproductive stage causes increase in sterile florets and decrease in plant height, root length, tillering ability and biomass.

- Then, the Styrofoam seedling float was suspended on the tray filled with distilled water for 2-3 days and then to nutrient solution for 4-5 days.
- After that, the 6 dsm^{-1} salt stress was given in two trays keeping remaining two as control and again after 5-6 days 12dsm-1 salt stress was given to the seedlings.
- When the susceptible variety (IR-29) scores 7; then the root, shoot and leaf was harvested.
- These harvested plant varieties were dried either in sun light or hot air oven.

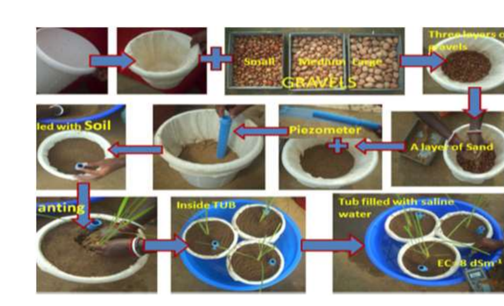
Pot preperation

- 20% volume of the soil was substituted by three different sizes of gravels.
- After using nylon mess in perforated pot, one layer of large followed by one layer of medium and small gravels were placed one after another at the bottom of the pot.
- One layer of sand was placed atop of the gravels. One perforated pipe (piezometer) was placed inside the soil with its opening outside the soil zone.
- The rest of the pot was filled with well ground soil mixed with medium size gravels.

Physiological parameters

The following physiological parameters can be taken -

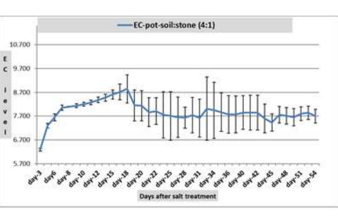
- Net chlorophyll estimation
- Na^{+} & K^{+} estimation
- Plant Biomass



Pot preparation in net house

Advantage

- Less fluctuation in Soil ECe and pH
- Lesser time to attain desired stress level
- More uniform stress imposition



Submergence Stress in Rice

- Plants require water for growth and development, but excessive water negatively affects their productivity and viability.
- Flash floods occasionally result in complete submergence of plants in agricultural and natural ecosystems.
- Submergence is a type of flooding stress and is defined as a condition where the entire plant is fully immersed in water (complete submergence) or at least part of the terminal shoot is remains above the water surface (partial submergence).
- Recurring floods in Asia cause poor crop establishment. Yields decline drastically when plants are completely submerged for a few days.

Working procedure

- Seeds of different tolerant and susceptible varieties of rice were pre- germinated and after 20-25 days.
- These seeds were transplanted in perforated pots.
- After 3 weeks before flowering, some of the prepared perforated pots were placed in plastic tub to allow saline water (having EC 8 dsm^{-1}) to go inside the pots from the bottom, and remaining in normal condition in net house till the tillering stage.
- The salinity level of the saturated soil was measured through piezometer using EC meter.

Screening Procedure

Field Screening in large submergence tanks

Tolerant Checks:
1. FR 13A
2. Swarna Sub-1

Susceptible Checks:
1. IR 42
2. Swarna

Parameters

The following physiological as well as molecular parameters may be taken –

- Na^+ & K^+ content in flag leaf
- Spikelet sterility
- Panicle length, weight & chaffyness

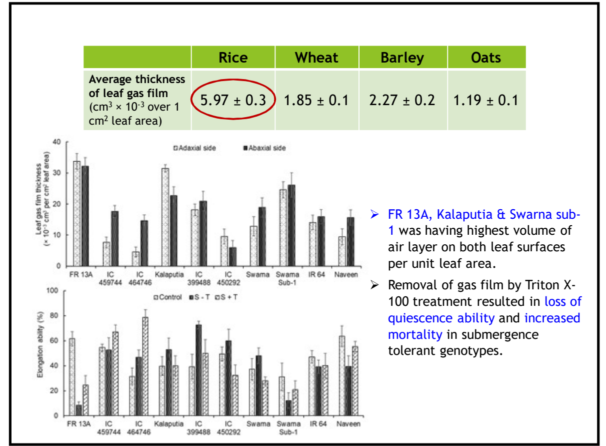
Pot Screening in smaller tanks

Tolerant Genotype
Swarna Sub-1

Susceptible Genotype
IR 64

Procedure

- Submergence study is preferably done in direct seeding method
- Seeds are sown directly in field or pots
- Plants are allowed to grow normally till 25 DAS
- Submergence stress is imposed by completely immersing the plants under water at ~ 25 DAS
- There should be at least 15 – 20 cm of water above the plant canopy
- There should be minimal disturbance in the tank water
- Stress is continued up to 12 – 14 days or the susceptible check dies completely



	Rice	Wheat	Barley	Oats
Average thickness of leaf gas film ($cm^3 \times 10^{-3}$ over 1 cm^2 leaf area)	5.97 ± 0.3	1.85 ± 0.1	2.27 ± 0.2	1.19 ± 0.1

Observations to be recorded

- Plant height before & after submergence
- Elongation ability
- Survival percentage
- Leaf senescence
- Leaf carbohydrate content

Leaf Hydrophobicity & Leaf Gas Film Thickness

- Leaf hydrophobicity & Leaf gas film thickness along with tissue porosity and leaf density can be used as surrogate trait for submergence tolerance
- Hydrophobicity can be measured through imaging or estimating epicuticular wax load

FR 13A | IC 450292 | Swarna Sub-1 | Swarna

0 h | 4 h | 24 h | 48 h | 96 h

HANDS ON PRACTICAL

ON

Development of C_4 Rice through transgenic approach.

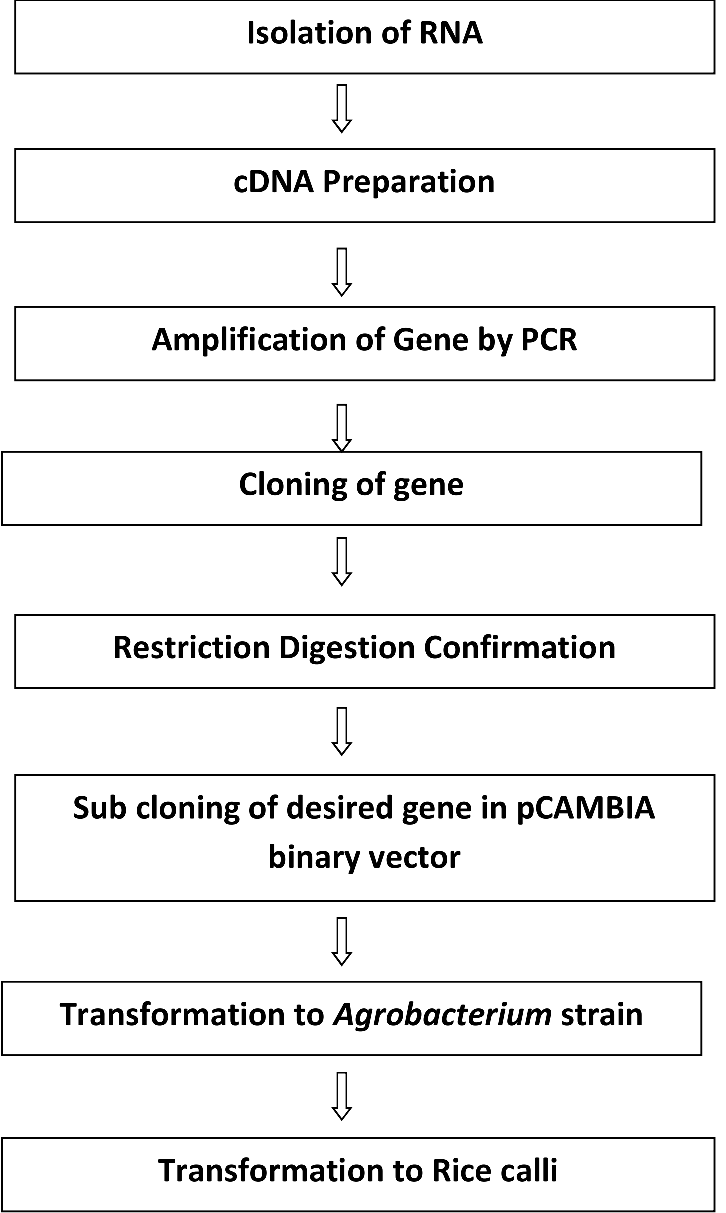
&

Introduction of Glycolytic pathway into rice chloroplast to bypass Photorespiration and increase Carbon fixation.

Awadhesh Kumar
Crop Physiology & Biochemistry
ICAR-NRRI, Cuttack

PRACTICAL 1: Development of C_4 Rice through transgenic approach.

Rice plant assimilate atmospheric CO_2 though the C_3 pathway of photosynthesis. Photosynthetically the rice plants are underachievers due to the dual activity (oxygenase and carboxylase) of Rubisco. Photorespiration process reduces net carbon gain and productivity of C_3 rice plants by as much as 50%. C_4 photosynthesis pathways overcome photorespiration and exhibit high rate of photosynthesis, fast growth and high efficiency in water and mineral use. Modern biotechnological tools are used to engineer C_4 traits in rice to enhance its photosynthetic efficiency and ultimately productivity. These are the steps to get transgenic rice plants.



Isolation of Total RNA from leaf samples

Young leaves are the best choice for isolation of total RNA from plant sample

↓

Disrupt around 100 mg of leaf tissue to fine powder by using mortar pestle with liquid nitrogen

Add 450 µl of Cell lysis buffer to the crushed sample and vortex it

↓

Transfer the lysate to the spin column for initial separation and centrifuged at 14000 rpm for 2 minutes

↓

Transfer the supernatant to new tube without disturbing the pellet

↓

Add 0.5 volume of ethanol to the supernatant and was mixed properly

↓

Transferred the sample to RNA binding column and centrifuge for 15 sec

↓

Add 700µl of wash buffer to the spin column and centrifuge for 15 sec

↓

Add 500µl of wash buffer to the spin column and centrifuge for 15 discard the flow-through and repeat this step twice

↓

Centrifuge the column at full speed to dry the membrane and add 30 - 40µl of RNase free water to elute purified RNA

↓

Quantification of total RNA was analyzed by using NanoDrop spectrophotometer

↓

Check RNA by electrophoresis in 1.2% formaldehyde agarose gel

cDNA preparation:

All traces of genomic DNA contamination are wipe out by *DNase* treatment of RNA samples prior to cDNA preparation. Prepare the reaction as per the table for DNase treatment.

Reaction mixture for DNase treatment of RNA samples.

Components [Concentration]	Volume used per reaction
Total RNA 10X Incubation Buffer DNase I recombinant, RNase free (1U/ µl) Stop solution	7.5 µl 1.0 µl 1.0 µl 0.5 µl
Total	10µl

Incubate the reaction mix at 37ºC for 10 minutes.

For inactivation of enzyme add 0.5 µl of EDTA to the tube and incubate it for another 10 minutes.

cDNA was synthesis using the Maxima cDNA synthesis kit (Thermo fisher)

Add the following components as per the table described below for cDNA synthesis.

Reaction mixture for cDNA preparation of RNA samples

Components [Concentration]	Volume used per reaction
DNase treated RNA 5X Reaction mix buffer Enzyme mix Nuclease free water	10 µl 4 µl 2.0 µl 4.0 µl
Total	**20µl**

Incubate this reaction mixture for 10 minutes at 25º C

Again incubate it at 50º C for 30 minutes

Incubate the reaction mixture at higher temperature [85ºC] for deactivation of enzyme.

NADP-ME C_4 chloroplastic enzyme plays a vital role in C_4 mechanism by converting the malate to pyruvate and releasing the CO_2 molecule. Decarboxylation of C_4 acids in the bundle sheath chloroplast is mainly catalysed by NADP-ME.

Amplification of C_4 gene Mallic enzyme [*ME*] by PCR

Asselembling of all the components of PCR reaction on ice and quickly transferring the reaction to a preheated thermo cycler to the denaturation temperature 98ºC is recommended for gene amplification.

For gene specific amplification of the *ME from the Setaria italica* (*Foxtail millet*) species*,* forward and reverse primers were designed from the genome sequence along with *Sa*lI and *Xba*I restriction enzyme respectively.

All components should be mixed prior to use. Reaction mixture is prepared by adding the following components as described in the table.

Reaction mixture for amplification of *ME* gene by PCR

Components	Volume/per reaction (µl)	Final concentration
cDNA Template (200ng/ µl)	1µl	200ng
10 µM ***SiMEG*** Forward	1.25 µl	0.5 µM
10 µM ***SiMEG*** Reverse	1.25 µl	0.5 µM
5X reaction buffer	5.0 µl	1X
5X GC enhancer buffer	5.0 µl	1X
10mM dNTPs	0.5µl	200µM
Q5Taq DNA Polymerase	0.25 µl	0.02 U/µl
RNase Free water	9.75 µl	-
Total	**25 µl**	**-**

For the amplification of desired fragment in the thermocycler (Eppendrof, USA), set the PCR programme for initial 30 second incubation at 98°C for complete denaturation, followed by 35 cycles consisting of 98°C for 0.10 min, 58°C for 30 secs, extension for 1min at 72ºC and final extension for 2 min at 72ºC.

Check the PCR product in gel documentation system after 1 % agarose gel electrophoresis.

Escherichia coli competent cells preparation by calcium chloride method

Prepare a primary culture of empty DH5α strain of *Escherichia coli* bacteria by inoculating 10 ml of Luria-Bertani (LB) broth and incubating at 37°C with shaking in an incubator shaker.

↓

Inoculate overnight grown primary culture (100 µl) into 10 ml of LB fresh medium as secondary culture and incubate it for about 1.5-2 hours in an incubator shaker at 37°C with shaking at 150rpm.

↓

Transfer the bacterial cells to a sterile ice cold oakridge tube (50ml) in a laminar air flow hood.

↓

Allow the culture to cool down by keeping the tube in an ice bucket for 30 minutes.

↓

Pellet down the cells by centrifuging at 6,000 rpm for 5 minutes at 4 °C and by discarding the supernatant.

↓

Resuspend the pellet in 5ml ice cold 100mM $CaCl_2$ and kept on ice for 5- minutes.

↓

Centrifuge the oakridge tube again for 5 min at 6,000 rpm at 4°C, the supernatant was discarded and resuspend the pellet in 1 ml of prechilled 100mM $CaCl_2$ solution buffer.

↓

Store the cell suspension overnight (about 16 hrs) in the ice bucket in refrigerator (4°C).

↓

Then aliquote the competent cells about 100 µl into prechilled sterile 1.5 ml tubes.

↓

Use directly the freshly prepared competent cell for transformation or store in -80°C after adding 50 µl of sterile glycerol to it for future use.

Cloning of *SiME (C_4)*gene in cloning vector

- Purify the PCR product or elute the amplified fragment from the gel.

- Ligate the PCR product and Vector by preparing the ligation mixture described in the table.

Ligation reaction mixture for ME cloning

Components	Volume/ reaction
ME PCR product	7 µl
Vector	1µl
10X ligation buffer (NEB ligase buffer)	1 µl
T4 *Ligase* (NEB)	1 µl
Total	**10.0 µl**

Gently mix the reaction mixture and incubate it at 16ºC temperature overnight in the thermo block (Genaxy). After incubation, use the mixture to transform in competent *E. Coli* bacteria (empty DH5α strain).

Bacterial transformation by heat shock method

Take the competent cells in the glycerol stock from -80°C and allowed to thaw on ice

Add entire ligation mixture with the competent cells in laminar air flow chamber. Incubate the micro centrifuge tube containing the bacterial cells and ligation mixture on an ice bucket for 30 minutes

Then pvovide a brief heat shock by transferring the tube to a floater in a pre-heated water bath maintained at 42°C and apply heat shock for exactly 90 seconds without any shaking

Immediately snap chill the tubes in an ice bath and allow the cells to chill for 2 minutes Add 250 µl of sterile LB broth to cells in the tube in laminar hood.

Incubate tubes for 1h at 37°C in an incubator shaker with shaking at 150 rpm to allow the bacterial cells to recover and express antibiotic resistance marker encoded by the plasmid.

↓

After the recovery period spread the culture in solid LB plate containing suitable selective antibiotic (*Ampicillin*), 100mM IPTG and 100mg/ml X-gal by spread plate method.

↓

Incubate the plates by inverting the plate upside down for overnight at 37°C. The IPTG and X-gal were used for the blue-white screening of transformed colonies.

PRACTICAL 2: Introduction of Glycolytic pathway into rice chloroplast to bypass Photorespiration and increase Carbon fixation.

Another possible strategy for enhancing carbon fixation and minimizing photorespiratory effect is the introduction of *Escherichia coli* glycolate catabolic pathway into rice chloroplasts to reduce the loss of fixed carbon and nitrogen and maintain photorespiration in plant. Five chloroplast-targeted bacterial genes encoding glycolate dehydrogenase (GLC-D,E,F), glyoxylate carboligase (GC) and tartronic semialdehyde reductase (TSR), have been amplified by PCR from *E. coli* gDNA and cloned in pGEMT-Easy vector. Gene specific primers with suitable RE sites were designed, PCR amplified and cloned into pGEM-3Z vector to design gene expression cassettes. Finally, two gene expression cassettes (*GC, GLC-D*) were inserted into binary vector (pCAMBIA-1304) at *Eco*RI+*Bam*HI and three (TSR, GLC-E & F) at *Eco*RI+*Xba*I sites to generate plant transformation vector. This will generate plants in which chloroplastic glycolate would be converted directly to glycerate. This would reduce, though may not eliminate, flux of photorespiratory metabolites through peroxisomes and mitochondria while increasing the rate of carbon fixation.

BACTERIAL DNA ISOLATION PROTOCOL

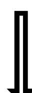
Transfer the bacterial culture to 1.5 ml microcentrifuge tube

Centrifuge for 1 minute at 10,000 rpm then discard the supernatant.

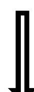
Transfer 180µl of Gram(-)buffer to the sample in the 1.5ml microcentrifuge tube then re-suspend the pellet by vortex

Add 20 µl of proteinase K then mix by vortex and incubate at 600 C for 10 min. During incubation invert the tube every 3 minutes.

Add 200µl of Lysis buffer to the sample and mix by vortex for 10 second. Incubate at 700 C for 10 minutes and invert the tube every 3 minutes.



Add 5µl of RNase A to the sample then shake vigorously and incubate at room temperature for 5 minutes.



Add 200µl of absolute ethanol to the sample and mix immediately by shaking

Place a GD column in a 2ml collection tube. Transfer the mixture to the GD column then centrifuge at 10000 rpm for 2 min.

Discard the 2ml collection tube containing the flow-through then place the GD column in a new 2 ml collection tube

Add 400µl of Wash buffer-1 to the GD column then centrifuge at 10,000 rpm for 30 seconds and discard the folw-through

Place the GD column back in the 2ml collection tube and add 600µl of wash buffer-2 to the GD column then centrifuge at 10,000 rpm for 30 seconds and discard the flow-through

Place the GD column back in 2ml collection tube and centrifuge again for 3 minutes at 10,000 rpm to dry the column

Transfer the dried GD column to new 1.5 ml microcentrifuge tube and add 100µl of Elution Buffer (pre-heated). Let it stand for 3 minute and centrifuge at 10,000 rpm for 30 seconds to elute the purified DNA.

Restriction Digestion of the cloned gene after plasmid isolation was performed for the confirmation by several restriction enzyme digestions. Colony PCR of the transformed colonies also confirms the cloning of the target gene. After the confirmation of cloning the genes were sequenced and analysed by NCBI blast.

Binary Vector constructs Preparation for *SiME* gene

Sub clone SiME gene in pCAMBIA-1301 binary vector in the downstream of promoter along with terminator for complete gene casstte formation. It needs sevveral step of sequential cloning. Several restriction digestion should be performed for confirmation of the clone. Transfer the gene construct to the agrobacterium strain by Fridge thaw method of transformation.

Agrobacterium mediated transformation to Rice calli

Take 21 days rice calli for *Agrobacterium*-mediated transformation method.

Use *Agrobacterium* strain *LBA4404* containing binary vector *pCAMBIA 1301* along with *SiME* gene construct for the transformation to rice calli.

Streak *LBA4404* containing *pCAMBIA 1301-SiME* gene construct in the LB agar plate containing *Rifampcillin* and *Kanamycin* antibiotic.

↓

Use two days old streak plate of *Agrobacterium* containing the binary vector gene construct for transformation.

↓

Scrap the bacterial cells from streak and dissolve in CIM infiltration media along with acetosyringone (100mM).

↓

Incubate the rice calli in the agro suspension and apply vacuum infiltration for 10-15 minutes.

Transfer the incubated calli to sterile filter paper and dried completely.

Transfer the infected calli to Co-Cultivation Media (CCM) containing CIM along with acetosyringone and keep it for 72 hours in dark.

After 3 days wash the calli with infiltration media containing cefotaxime (200mg/ml) and dry it completely.

↓

Culture the transformants on selection media plate supplemented with 50 mg/L hygromycin B. After three round of second the calli are transferred to regeneration media

In-Planta method of transformation

Dehusk the seed

Surface Sterilisation

Soak it for one hour at 370c

Transfer the seeds to the filter paper and let it dry slightly

Pierce the seed in very fine needle

Vaccuminfilteration with the agro solution for 20 minutes

Let it dry in the filter paper completely

Transfer the infected seeds to co-cultivation mediaplate(Agar media with Acetosyringon)

Incubate for three days

Wash the seed in double distilled water

After three days transfer the seeds to selection plate

Let it grow for ten days

Transfer the selected plants to rooting media

Transfer to soilrite

Transfer the plants to pot for hardening

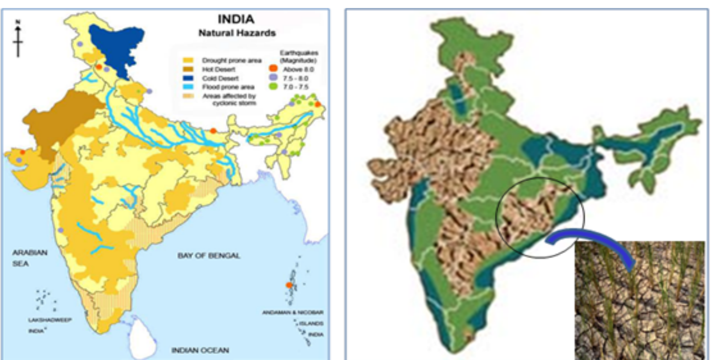
SCREENING TECHNIQUES FOR DROUGHT TOLERANCE IN RICE

Padmini Swain

Crop Physiology and Biochemistry Division
ICAR-National Rice Research Institute, Cuttack, Odisha
e mail: pswaincrri@gmail.com

Introduction

Rice has been grown under diverse range of ecosystems and gets exposed to different environmental stresses like drought, salinity, submergence, cold etc., wherein drought constitutes an important yield limiting determinant. Out of 43.3 million ha area under rice in India, drought is one of the major abiotic constraints in around 7.3 million ha of rainfed upland and rainfed lowland situations. Eighteen percent of total rice area of India and 20% of Asia are drought prone. The irregularities in South-west monsoon do result in moderate to severe drought in rainfed rice growing areas especially in eastern India.



Drought prone areas in India

Screening for drought tolerance:

To identify rice germplasm lines with built in tolerance to vegetative and reproductive stage drought, large no. of rice germplasm including upland rice, lowland rice, deep water rice, wild rice, aromatic rice and fixed lines are being screened at NRRI, Cuttack under field condition during dry (*rabi*) season. Details of protocol standardised and followed are described below for convenient and easy way of screening varieties/germplasm lines.

Experimental sites:

Generally for large scale screening experiments are to be conducted under field condition during dry season where interference of rain is negligible during the cropping period. Wherever controlled facility like Rain out shelter is available, screening can be done in normal wet (*kharif*) season also. Depending on the soil type the irrigation/stress schedule is to be managed.

Screening environments:

1. Artificially created stress - in the normal growing season (*kharif/wet*)
2. Managed water deficit - in a non growing season (*rabi/dry*)

Soil properties:

Data on soil type, pH, EC, and available NPK content is to be measured. Soil moisture content at -0.03, -0.05, -0.10, -0.50 and -1.50 MPa metric potential to be measured to make a soil moisture release curve. The field to be properly tilled and laser leveled/manually levelled in order to avoid huge variation in soil moisture content within the experimental unit.

Experimental design:

If large no. of varieties (>1000 entries) are to be sown in the field, augmented design is to be followed. The field is to be divided into blocks and the varieties are to be divided in equal number for each block. Better to use more no. of tolerant and susceptible checks. Tolerant and susceptible checks are to be randomised in a set of 10/20 lines each and the susceptible check can also be put in the border lines.

Seed Sowing:

Varieties are to be dry direct seeded with 4-5 seeds per hill. Spacing of 20 cm (row to row) and 10 cm (plant to plant) is to be maintained for each experiment. The line length will be 3 m single line per genotype or 1.5m x 2 lines per genotype depending on the area of the field. After 15 to 20 days after sowing, thinning/gap filling is to be carried out to maintain uniform plant population.

Stress Management:

Plants are to be grown for 28days (4 weeks) after germination with sprinkler irrigation in 3-4 days interval and then it is to be withdrawn for 30 days or beyond, till the susceptible check shows permanent wilting and maximum number of lines show leaf rolling and tip drying symptoms. Phenotypic observations will be recorded during the stress period and then the plants to be will be recovered by re-watering.

For vegetative stage stress, the stress will be imposed at active tillering stage (4 wk stage) and for reproductive stage stress, the stress will be imposed at booting stage. For reproductive stage stress separate protocol is to be followed and described below.

During the period of stress *peizometer* (perforated PVC pipes) are to be fixed to monitor ground water table depth every day (more nos. preferable for bigger plots).

Large scale field screening for drought tolerance during dry (*rabi*) season

Screening facility under Automated Rain-out shelter during wet (*Kharif)* season

Soil sampling for soil moisture content:

Soil moisture to be taken at 15 cm and 30 cm depth at 5 days/weekly interval after suspension of sprinkler irrigation till the susceptible check shows permanent wilting. Soil sample to be taken in a zigzag fashion from the whole field at least from 2 different places in each block. Soil sample is to be collected carefully by augur and kept in aluminum boxes, fresh weight of the soil + box is to be taken and then the soil to be dried in an oven at 100^0C at least for 48 hrs. Then dry soil weight is to be taken with the box and by deducting the blank box weight the SMC% to be calculates as

$$\text{SMC\%} = \frac{(\text{Soil fresh wt- soil dry wt}) \times 100}{\text{Soil fresh wt}}$$

Recording for soil moisture tension:

Soil moisture tension (soil metric potential) to be recorded in similar fashion by installing *tensiometer* tubes at 30 cm or 45 cm soil depth in both control and stress treatment blocks. At least 10 *tensiometers* will be installed to cover the experimental units. When soil moisture tension reaches -40 to -50 kPa at 30 cm soil depth for vegetative stage stress and -50 to -60 kPa for reproductive stage stress, the plot is to be re-irrigated.

Phenotypic observations:

Some of the important morpho-physiological, phonological and biochemical observations to be taken during stress period

Leaf rolling and death score: Leaf tissues may die (showing desiccation) because of extreme loss of water or because of heat stress when the leaf temperature rises as a result of inadequate transpirational cooling. Leaf rolling and tip drying (drought score) and recovery data to be recorded following IRRI SES method, 1 to 9 scales (IRRI, 1996). The varieties/lines with early leaf rolling after suspension of sprinkler irrigation showed higher score for drought tolerance (7-9) will be considered as susceptible ones. Varieties/lines with delayed leaf rolling (SES '1-3') and recovered faster after re-watering will be considered as tolerant ones.

Table: Standard Evaluation System (SES - IRRI, 1996) for rice leaf rolling, tip drying (drought score) and recovery score at vegetative stage (IRRI, 1996).

Leaf rolling score at vegetative stage		**Tip drying score at vegetative stage**		**Recovery score after releasing stress(24 hrs, 72 hrs and 10 days after stress)**	
Scale	**Symptoms**	**Scale**	**Symptoms**	**Scale**	**Recovery**
0	Leaves healthy	**0**	No symptoms	**1**	90-100%
1	Leaves start to fold (shallow)	**1**	Slight tip drying	**3**	70-89%
3	Leaves folding (deep V-shape)	**3**	Tip drying extended up to 1/4	**5**	40-69%
5	Leaves fully cupped (U-shape)	**5**	One-fourth to 1/2 of all leaves dried	**7**	20-39%
7	Leaf margins touching (O-shape)	**7**	More than 2/3 of all leaves fully dried	**9**	0-19%
9	Leaves tightly rolled	**9**	All plants apparently dead. Length in most leaves fully dried		

Leaf sample collection for different Physiological and biochemical estimations:

Top-most fully expanded leaf (in case of vegetative stage stress) or flag leaf will be sampled between 11:00 to 14:00h. At least three or more plants will be sampled per genotype. Half of the leaf will be used for RWC, while rest half of the leaves will be used for leaf water potential, cell membrane stability chlorophyll, proline and measurements. Chlorophyll and proline will be expressed on the fresh/dry weight basis of leaf.

Relative water content (RWC):

RWC to be measured following the method of Barrs and Weatherley (1962).

1. Cut fresh leaf slices into 1-1.5 mm size
2. Weigh the samples immediately to record the fresh weight of the sample.
3. Hydrate the samples to full turgidity by floating on de-ionized water in a close petridis for 8 hr under normal room light and temperature.
4. After 8 hours take leaves out of water and remove any surface moisture quickly and lightly with filter paper and immediately weigh to obtain fully turgid weight.
5. Oven dry samples at 80ºC for 24 hr and weigh to determine dry weight of the sample and then calculate RWC by following the formula

$$\text{RWC (\%)} = \frac{[(\text{Fresh weight}) - (\text{Dry weight})]}{[(\text{Turgid weight}) - (\text{Dry weight})]} \times 100$$

Leaf water potential:

Fully expanded youngest leaf to be collected in polyethylene bag at the midday (12hrs to 14.0hrs) and kept in ice bucket. Cut small leaf disc with the help of a punching machine and keep inside the leaf chambers attached to the *Water Potential System, Psypro, WESCOR* (Germany). The chamber with the leaf disc will be left for 5 minutes to stabilize the temperature, and then switch on the machine to record the water potential of leaf discs. Eight observations can be taken at a time within 15 minutes.

Membrane stability index:

Cell membrane stability (CMS) is to be measured from electrolyte leakage from control and drought stressed leaf tissue following Blum and Ebercon (1981). Leaf samples to be collected and washed three times in deionized water to remove electrolytes adhered on the surface, then kept in a capped vial (20 ml) containing 10 ml of deionized water and incubated in the dark for 24 h at room temperature. The conductance to be measured with a conductivity meter. After the first measurement the vials to be autoclaved for 15 min to kill the leaf tissue and release the electrolytes. After cooling, the second conductivity reading to be taken. These two measurements are to be carried out individually for all the samples from both the control and stress treatments. The control will give a measure of leakage solely due to the cutting and incubation of leaf discs. The conductance of the stress sample is a measure of electrolyte leakage due to water stress and is assumed to be proportional to the degree of injury to the membranes. CMS will be calculated as the reciprocal of cell-membrane injury.

CMS%= [(1-(T1/T2))/(1-(C1/C2))]´100, where,

T and C refer to the stress and control samples, respectively; and

1and 2 refer to the initial and final conductance readings, respectively.

Water use efficiency & Transpiration efficiency estimation:

Water use efficiency of selected genotypes is to be studied following the method described by Kholova *et al* (2010).

Transpiration efficiency (TE) will be measured in well watered WW and water stress WS plants and calculated as the production of biomass per amount of water transpired during the dry down as:

TE = (final harvested biomass - pre dry down biomass)/water transpired

Water transpired is the sum of daily transpiration measured in the dry down, assessed by regular weighing of pots and recording of water added. The final harvested biomass is that of WW and WS at the end of the dry down.

However, other than this method advanced methods are also followed like carbon/oxygen isotope discrimination methods:

The flag leaf/grain samples to be analyzed for ^{13}C isotope discrimination to measure the WUE and $\delta^{18}O$ to be measured from leaves as a surrogate for mean transpiration rate (Sheshshayee *et al*. 2005).

Chlorophyll content and Chlorophyll stability index:

Total chlorophyll content is to be estimated by the method described Arnon (1949).

1. 25 mg of leaf samples (fully matured leaf) to be dipped in 10 ml of 80% acetone in a graduated glass tube.
2. Incubate the glass tubes in dark at 4°C for 48 hrs.
3. Take absorbance at 645 nm and 663 nm after 48 hrs.
4. Quantify the total chlorophyll, chlorophyll ‘a’ and chlorophyll ‘b’ content from the 80% acetone extract by following the calculation:

Total chlorophyll (µg/ml) = 20.2 (A_{645}) + 8.02 (A_{663})
Chlorophyll a (µg/ml) = 12.7 (A_{663}) - 2.69 (A_{645})
Chlorophyll b (µg/ml) = 22.9 (A_{645}) - 4.68 (A_{663})

where A_{663} is the solution absorbance at 663 nm andA_{645} is the absorption at 645.

The Chlorophyll content was expressed as mg g^{-1} fresh weight.

Chlorophyll stability index (CSI) to be calculated as the percent of chlorophyll content under stress as compared with that under normal conditions.

Calculation: CSI = $\frac{\text{chlorophyll content under stress}}{\text{chlorophyll content under normal condition}} \times 100$

Proline content:

Proline acts as an osmo–regulator and its concentration in many plants or tissues exposed to a variety of abiotic stresses has been frequently studied. Proline content to be measured follwing the method of Bates *et al*. 1973.

Fresh leaf sample of 0.5g was homogenized in 10ml of 3% aqueous sulphosalicyclic acid. The homogenate was filtered through Whatman No. 2 filter paper. Reaction mixture was prepared by adding 2ml of filtrate, 2ml of glacial acetic acid and 2ml acid ninhydrin (1.25g ninhydrin in 30ml glacial acetic acid and 20ml 6M phosphoric acid). The mixture was heated to boiling for 1 hr. Reaction was terminated by placing the tubes in ice bath. 4 ml of toluene was added to the reaction mixture and stirred for 20-30 sec. The red colour toluene layer was separated and then its intensity was measured at 520nm using S*pectrophotometer* (Genesys 200). Standard curve was prepared by running a series of standard with pure proline. Poline amount in the test sample from the standard curve was calculated using the following formula:

µmoles per g tissue = $\frac{\mu g \text{ proline/ml} \times \text{ml toluene} \times 5}{115.5 \times \text{g sample}}$

Where 115.5 is the molecular weight of proline

Gas exchange parameters

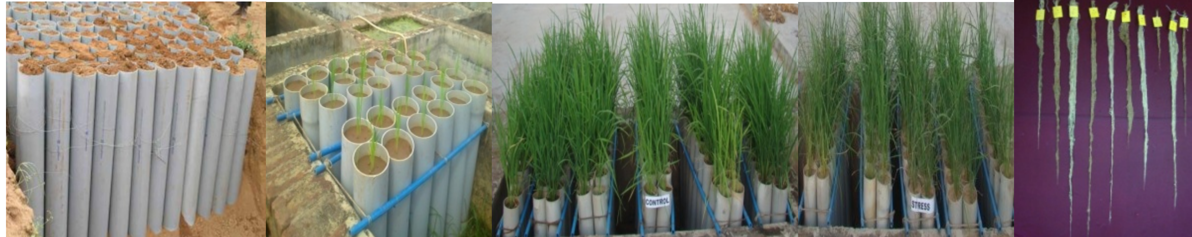
Gas exchange parameters were measured in the fully expanded leaf second from the top of the plant by using a portable *Infrared Gas Aalyzer* (IRGA) (LI-6400; LI-COR, Lincoln, NE). The measurements were made at an ambient CO_2 concentration of 400 µmol mol^{-1} and photosynthetic photon flux density of 1,500 µmol m^{-2} s^{-1} by using LICOR light source and a chamber temperature of 28°C $\pm$ 0.5°C. The parameters net photosynthetic rate (*A*) and transpiration rate (*T*) were used to calculate the instantaneous WUE (ratio *A*/*T*), which is the amount of CO_2 fixed per unit amount water lost by transpiration.

Chlorophyll fluorescence

For chlorophyll fluorescence measurement the same intact leaf was used. Chlorophyll fluorescence was measured using a *Plant Efficiency Analyzer* (Handy *PEA*, Hansatech Instruments Ltd., Norfolk, UK). To measure the Chl a fluorescence transients, leaves were maintained in darkness for 30 min and data were recorded from 10 µs up to 1 s with data acquisition every 10 µs for the first 300 µs, then every 100 µs up to 3 ms and later every 1 ms. The maximal intensity of the light source, providing an irradiance saturating pulse of 3500 µmol photons/m^2/s, was used.

Phenotyping for root morphological traits:

Since root traits are associated with drought tolerance under field condition, germplasm lines differing in their response towards drought can also be evaluated for root traits in PVC pipes under moisture stress at vegetative stage. PVC pipes of 75cm/1.0mt long and 20cm diameter to be filled with equal amount of soil and single seed of a variety to be sown and irrigated. 28 days after germination irrigation to be stopped and after 20days of stress or when the susceptible check showed rolling symptom, the tubes to be put in water tanks for 24 hrs. The soil in the pipe will be loosen and the root with soil to be taken out carefully and washed with jet pipe so that any root will not be broken. The entire root to be taken and different root traits like maximum root length (MRL), total root length (TRL)(can be measured by root scanner), shoot length (SL), root volume, root dry weight (RDW), shoot dry weight (SDW), are to be recorded and other root traits can be extrapolated from these basic data.



ROOT TRAIT STUDIES UNDER PVC PIPES

Protocol for Reproductive stage stress:

For reproductive stage drought screening, varieties to be dry direct seeded in the field. The seedlings to be allowed to grow up to panicle initiation stage with normal irrigation in 3 days interval with no standing water. Then irrigation to be stopped till 15 days after flowering. Soil

moisture content (SMC) and soil moisture tension (SMT) during the stress period to be monitored. SMC% to be 10-12% at 30cm depth and SMT to be -50 to -60 KPa at 30 cm depth. Irrigation to be given after releasing stress and to continue in 5 days interval till maturity with surface irrigation only. Days to 50% flowering (DFF), plant height, ear bearing tiller (EBT), grain number, chaff no, grain yield, total biomass, harvest index (HI), panicle length, spikelet fertility are to be recorded.

Phenology: Days to 50% flowering, days to physiological maturity, flowering delay and reduction in grain filling duration to be recorded.

Spikelet fertility/grain filling%: Number of filled and unfilled spikelets to be recorded at physiological maturity to calculate spikelet fertility (%) both in control and stress treatments (Fischer *et al*. 2007).

Yield and its components: One meter row or 10 hills to be harvested at maturity. Plant number, tillers per plant, panicles per plant, total biomass, grain yield and 1000 grain weight data to be recorded.

Relative yield reduction (RYR) and Drought susceptibility index (DSI) (Fischer and Maurer, 1978) analysis to be used to rank the genotypes for drought tolerance in yield.

$$\textbf{RYR\%} = \frac{\text{Yield under control- Yield under control}}{\text{Yield under control- Yield under control}} \times 100$$

$$\textbf{DSI} = \frac{\text{1- (yield under stress/yield under control)}}{\text{(mean yield under stress/ mean yield under control)}}$$

Or DSI= $(1-Y_{WS}/Y_{WW})/D$

D=1-(Experimental mean under WS /Experimental mean under WW)
Y_{WS}= Grain yield of the genotype under vegetative stage drought stress
Y_{WW}= Grain yield of the genotype under well water condition

High values for DSI represent drought susceptibility (Winter *et al.* 1988). DSI for grain yield or any other trait **close to or below 1**, indicates the relative tolerance of that trait to drought. Based on the value and direction of desirability, ranking was done for different genotypes as: highly drought tolerant (DSI<0.50), moderately drought tolerant (DSI: 0.51-1.00) and drought susceptible (DSI>1.00). Yield under stress and DSI are negatively correlated.

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ASSESSMENT OF QUALITY PARAMETERS IN RICE GRAIN

Nabaneeta Basak, Gaurav Kumar
Crop Physiology and Biochemistry Division
ICAR-NRRI, Cuttack, Odisha-753006

Rationale:

As more countries become self-sufficient in rice production, there has been an increase in the demand for better quality rice. Being a multifaceted trait, quality plays a foremost role in dictating the consumers' choice and marketability. Grain quality is not exclusively a varietal characteristic but also depends on the crop management practices, processing and handling systems. In the context of climate change further emphasis is given on the production of nutrient packed rice grains to cater to the nutritional needs of the millions of people predominantly dependent on rice.

Objectives:

To understand the different factors that affect grain quality and also how to measure the physicochemical parameters that affect quality.

Grain Quality Characteristics:

Rice grain quality encompasses various physical and chemical characteristics that may be either genetic or acquired (Rice Quality Training Manual, IRRI).

The *genetic properties* include:

- chemical characteristics (gelatinization temperature, apparent amylase content, gel consistency, alkali spreading value and aroma),
- shape,
- size,
- color of grain,
- chalkiness,
- bulk density,
- thermal conductivity,
- equilibrium moisture content and

- flowability.

The *acquired properties or environmental factors* include:

- moisture content,
- grain purity,
- physical and pest damage, cracked grains,
- presence of immature grains and
- milling-related characteristics (milling and head rice recoveries, grain dimensions, whiteness, milling degree and chalkiness).

Quality Characteristics of Rice:

The quality characteristics of milled rice are classified both physically and chemically.

Physical characteristics:

Hulling(%):

Removal of external husk is called hulling. Hulling percentage is calculated by weighing the brown rice after hulling and expressed in % basis.

Milling (%):

The degree of milling or percent brown rice removed as bran affects the level of recovery and influences consumer acceptance.

The brown rice (Hulled rice) collected after hulling are subjected to milling i.e. removal of bran layer for 60 sec. Then polished rice grains are graded according to their recovery:
(1) Head Rice: The unbroken white rice (2) Broken rice: Half or less than half of original size. After grading head rice and brown rice are separately weighed. The milling % is calculated by adding Head rice and broken rice together.

Head rice percentage:

The head rice percentage is the volume or weight of head grain or whole kernel in the rice lot. Head rice normally includes broken kernels that are 75-80% of the whole kernel. High head rice yield is one of the most important criteria for measuring milled rice quality.

Whiteness:

This characteristic is a combination of varietal physical characteristics and the degree of milling. During whitening, the silver skin and the bran layer of the brown rice is removed. Polishing is undertaken after whitening to improve the appearance of the white rice.

Chalkiness:

Grain appearance is largely determined by the endosperm opacity and this is commonly classified as the amount of chalkiness. Though chalkiness disappears upon cooking and has no direct effect on cooking and eating qualities, excessive chalkiness downgrades the quality and reduces milling recovery.

Kernel length (KL):

The length of head rice is measured in mm by machine or scale is called KL.

Kernel breadth (KB):

The breadth of head rice is measured in mm by machine or scale are called KB

L/B ratio:

The ratio of whole grain length and breadth is called L/B ratio.

***Grain size & shape*:**

Grain size and shape are important criteria of rice quality for developing new varieties for trade and hence for quality evaluation. In India, Ramiah's classification (Govindaswami, 1985) is followed and is given below (Table 1):

Table 1: Rice grain classification followed in India

Grain type	Milled Grain Length (mm)	Length : breadth ratio
Long slender (LS)	≥ 6 mm,	≥ 3
Short slender (SS)	< 6 mm	≥3
Medium slender (MS)	< 6 mm	2.5 to 3.0
Long bold (LB)	≥ 6 mm	< 3
Short bold (SB)	< 6 mm	< 2.5

Chemical characteristics:

The cooking quality of rice grains is determined by their *alkali spreading value* (ASV), *gelatinization temperature* (GT) *water uptake* (WU) value , *volume expansion ratio* (VER), *kernel length after cooking* (KLAC), *elongation ratio*(ER), *gel consistency* (GC) and *apparent amylose content* (AC).

Kernel Length after Cooking:

KLAC measure lengthwise elongation during cooking. Rice that exhibits better lengthwise elongation during cooking commands high price.

- Polished rice is added to distilled water in a cooking tube. The increase in volume after adding sample is noted. It is cooked in boiling water. The cooked rice is then pulled out and the length is measured and expressed in mm.

Elongation Ratio:

Principle: It is the ratio of kernel length after cooking to the kernel length before.

Volume Expansion Ratio:

It is a measure of the increase in volume of rice after cooking.

- 50 ml water is taken in a measuring cylinder. To it, the cooked rice after taking kernel length is added and total volume increase is noted. It is expressed as the ratio of increase in volume after cooking to the increase in volume before cooking.

Water Uptake:

WU value is a measure of the volume of water absorbed by 100g of grains and also an indicator of gelatinization.

- Polished grain is added to water in a cooking tube and soaked for half an hour. It is then kept in water bath at 77ºC for 45 min along with blank. The water is then decanted and allowed to stand for 1 hr. Thereafter the volume (ml) of water above residue is recorded. Blank containing only water was also processed in the same way.
- The water uptake value is determined by subtracting the volume (ml) of water in sample from blank and expressed in terms of ml of water absorbed per 100g rice grain.

Gelatinization Temperature:

The temperature, at which the starch granules swell in water irreversibly losing their crystallinity, is determined by gelatinization temperature.

Gelatinization temperature is estimated by the extent of alkali spreading (ASV) and clearing of milled rice soaked in 1.7% KOH at room temperature or at 39ºC for 23 hours and looking for disintegration of grains, on a 1-7 scale.

The degree of spreading is measured using a seven-point scale as follows:

1. grain not affected
2. grain swollen,

3. grain swollen, collar incomplete and narrow,
4. grain swollen, collar complete and wide,
5. grain split or segmented, collar complete and wide,
6. grain dispersed, merging with collar; and
7. grain completely dispersed and intermingled.

Alkali spreading value corresponds to gelatinization temperature as shown in Table 2.

Amylose Content:

Starch makes up about 90% of the dry matter content of milled rice. Starch is a polymer of glucose and amylose is a linear polymer of glucose. The amylose content of starches usually ranges from 15 to 35%. High amylose content rice generally cook dry, are less tender, and become hard upon cooling. In contrast, low-amylose rice cooks moist and sticky. Intermediate amylose rice is preferred in most rice-growing areas of the world, except where low-amylose japonicas are grown.

It is normally measured by its color reaction with iodine.

- To the sample, 1 ml ethanol and 9 ml of NaOH is added and boiled on water bath and volume made upto 100ml.
- To 5ml of the extract, 1 ml of 1N acetic acid and 2ml of iodine reagent is added and kept in dark for 20 min.
- Volume is made upto 100ml and absorbance measured at 620nm.

Gel consistency:

Gel consistency measures the tendency of the cooked rice to harden on cooling. Within the same amylose group, varieties with a softer gel consistency are preferred, and the cooked rice has a higher degree of tenderness. Harder gel consistency is associated with harder cooked rice and this feature is particularly evident in high-amylose rice. Hard cooked rice also tends to be less sticky.

Gel consistency is determined by heating a small quantity of rice in a dilute alkali. This test differentiates the consistency of cold 5.0% milled rice paste.

Table 2. Classification of milled rice according to their apparent amylose content, alkali spreading value, gelatinization temperature and gel consistency

Property	**Type or Class of rice**	**Range**
Apparent amylose content	Waxy	0 – 2%
	Very low	2-9%
	Low	10 - 20%
	Intermediate	20 - 25%
	High	25 - 33%
Alkali spreading value	Low GT	6 - 7 (GT < 70^0C)

	Intermediate GT	4 - 5 (GT 70-74^{0}C)
	High Intermediate GT	3
	High GT	1 - 2 (GT $> 74^{0}$C)
Gel consistency	Hard	25 - 40 mm
	Medium	41 - 60 mm
	Soft	61 - 100 mm

Contact:

ICAR-National Rice Research Institute
Cuttack-753 006, Odisha India
Phone: 91-671-2367768-83 | Fax: 91-671-2367663
Email: crrictc@nic.in | director.nrri@icar.gov.in
URL: www.icar-nrri.in