MATERIAS AND
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MATERIALS

Fungicide

The fungicide BAAN (5-methyl-1, 2, 4–triazole (3, 4-b), benzo-1, 3-thiazole), was obtained from local pesticide dealer.

It is a pale brown wettable powder, soluble in water, chloroform, methanol, acetone and other agricultural solvents. The recommended dosage of this fungicide ranges from 0.6g/lt for foliar spray per hectare and 2g/kg of seeds for seed treatment. Different concentrations of fungicide solution were prepared with distilled water by dissolving 0.1(which contains about 0.075g tricyclazole as active ingredient), 0.2 (0.15g tricyclazole) and 0.3g (0.225g tricyclazole) of fungicide in 100ml of distilled water.

Fig. 4.1 : Fungicide

Seed Samples

Seed samples of eight cultivars of rice (*Oryza sativa* (L.) namely, Thanu, B.R.2655, MTU1001, MTU1010, IET7575, IR30864, KMR-3R and KRH-2 were procured from Zonal Agricultural Station, V.C. Farm, Mandya.
Fig. 4.2 : Rice Cultivars
Table 4.1: Salient Features of the Rice cultivars used for the study

<table>
<thead>
<tr>
<th>Variety of paddy</th>
<th>Inbred</th>
<th>Duration of paddy to harvest</th>
<th>Yield per acre in quintals</th>
<th>Special features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thanu</td>
<td>Mandya Vijaya x Mukthi</td>
<td>130 -135</td>
<td>26 -28</td>
<td>Good grain, quality,</td>
</tr>
<tr>
<td>B.R.2655</td>
<td>Imported from Bangla</td>
<td>140-145 (L)</td>
<td>30-35</td>
<td>Good grain, quality, high yielding</td>
</tr>
<tr>
<td>MTU1001</td>
<td>MTU 5248 x MTU 7014</td>
<td>130-135</td>
<td>28-30</td>
<td>High yield, BPH tolerant, suitable for beaten rice</td>
</tr>
<tr>
<td>IR30864</td>
<td>IR-1738 x IR 7801 x IR-46x Kavaloo</td>
<td>130-135</td>
<td>28-30</td>
<td>High yield</td>
</tr>
<tr>
<td>IET7575</td>
<td>Sonax Manohar Sali</td>
<td>130-135</td>
<td>25-28</td>
<td>High yield, BPH tolerant</td>
</tr>
<tr>
<td>KRH-2</td>
<td>IR-58025 A x KMR-3RA</td>
<td>130-135</td>
<td>40 - 45</td>
<td>High yield</td>
</tr>
<tr>
<td>MTU1010</td>
<td>Krishnaveni x IR 64</td>
<td>120</td>
<td>40 - 45</td>
<td>High yield, BPH tolerant.</td>
</tr>
<tr>
<td>KMR-3R</td>
<td>Parental line of KRH-2</td>
<td>130</td>
<td>25-30</td>
<td>Bold grain</td>
</tr>
</tbody>
</table>

Locally grown eight cultivars of rice were selected for the present study. Seed samples were tested for their sensitivity to fungicide BAAN by conducting preliminary germination experiments.

**Chemicals**

Acetone, Bovin serum albumin, Sodium Potassium Tartrate, Sodium Carbonate, Sodium Hydroxide pellets, Folin–Ciocalteu reagent, concentrated Hydrochloric acid, 3,5-Dinitrosalicylic acid, Hydrogen peroxide, Mercury Chloride, Manganese Sulphate, Starch, Copper Sulphate, Sodium bicarbonate, concentrated Sulphuric acid. Brucine sulphate, Sulfanilic acid, Sodium Chloride, Potassium Nitrate, Nitric acid, Perchloric acid, Ammonium Molybdate, Ammonium Vandate, stock solutions of Copper, Zinc, Iron, Manganese Sulphate, Potassium Chloride, Calcium Chloride.
Methods

Seed Germination Test

The germination studies were carried out according to the between paper method recommended by International Seed Testing Association (ISTA, 2003).

The healthy seeds of paddy samples were selected and surface sterilized with 0.1% mercuric chloride for five minutes and rinsed with distilled water 5 to 6 times. The concentrations of fungicide viz., 0.1, 0.2, and 0.3% were prepared by diluting formulated grade with distilled water. The seeds were soaked for 24 hours in different fungicide concentrations, seeds soaked in distilled water served as control. Twenty seeds of each cultivar were equidistantly arranged in each germination towel rolled, and kept for germination. Three replicates were maintained for each concentration including control. The experiments were repeated three times at regular time intervals, thus representing a total sample of 200 seeds for each concentration.

The paddy seeds were germinated at 28±2°C. The seeds germinated were counted on 15th day and the germination percentage was calculated. The protrusion of radicle through the seed coat was taken as a criterion for germination.

Morphological Parameters

On 15th day of germination, various morphological parameters like length of shoot and root, fresh and dry weights, percent germination, and seedling vigour were recorded according to ISTA standards (Anonymous, 1985). The experiments were repeated three times at regular intervals. Based on the average of three set of experiments in each treatments including control different parameters were calculated as described below.
Fresh Weight and Dry Weight (grams)

All the measured seedlings were weighed to record the fresh weight. The seedlings were then dried in a hot air oven at 80°C for 48 hours and their dry weights were noted with the help of digital single pan balance.

Germination Percentage

Germination refers to the initial appearance of the radicle by visual observation. It was calculated by using the formula prescribed by ISTA (2003).

\[
\text{Germination } \% = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds sown}} \times 100
\]

Vigour Index

Vigour index of the samples was calculated by the formula suggested by Abdul Baki and Anderson (1973).

\[
\text{Vigour index (VI)} = (\text{mean root length} + \text{Mean shoot length}) \times \text{Percentage Germination}
\]

Tolerance index (percent)

Tolerance index was calculated by using the formula given by Turner and Marshal (1972).

\[
T \ I = \frac{\text{Longest root in treatment}}{\text{Longest root in control}} \times 100
\]
Percent of phytotoxicity

The percent phytotoxicity of the seedling due to fungicide treatment was calculated by the formula suggested by Chiou and Muller (1978).

\[
\text{Percent phytotoxicity} = \frac{\text{Radicle length of control} - \text{Radicle length of treated sample}}{\text{Radicle length of control}} \times 100
\]

Biochemical Parameters

15\textsuperscript{th} day old seedlings were used to estimate various bio-chemical parameters like total protein content, total chlorophyll, chlorophyll-a, chlorophyll-b, content and specific activity of two enzymes like \(\alpha\)-amylase and catalase.

Total Protein Content (mg/gram fresh weight of tissue)

The total protein content was determined following Lowry \textit{et al.}, (1951) method.

Sample Preparation

0.5g of the sample was homogenized using 5ml phosphate buffer in pestle and mortar. The homogenized mixture was centrifuged at 10,000 rpm for 20 min in refrigerated centrifuge. The supernatant was used for protein estimation.

Estimation

About 1 \(\mu\)l of each sample was taken into labeled test tubes. The volume of the sample was made upto 1ml by using distilled water. About 5ml of the alkaline Copper sulphate solution was added to each tube, mixed well and allowed to stand for 10 minutes. Then 0.5ml of Folin – Ciocalteu reagent was added, mixed well and
incubated at room temperature in dark for 30 minutes. Blue colour develops. Absorbance was read at O.D 660nm. The standard graph was plotted using Bovine serum albumin and amount of protein in sample was calculated.

**Estimation of Chlorophyll (mg/gram fresh weight of tissue)**

The leaves of 15th day old seedlings were used to estimate chlorophyll content by the method of specific absorption co-efficient of Mckiney (1940).

**Preparation of Sample**

0.5g fresh leaves were homogenized in mortar and pestle with 20ml of 80% acetone. The extract was filtered using Whatman’s no.1 filter paper. The residues were re-extracted with 5ml of 80% acetone each time, until it became colourless. The supernatant was used for chlorophyll determination and optical density was measured at 660nm and 600nm using colorimeter. The chlorophyll content was determined by using the following formula.

\[
\text{Chl.’a’ mg/g fresh weight of the tissue} = \frac{12.7 \times (\text{O.D at 660}) - 2.69 \times (\text{O.D at 600})}{1000 \times W} \times V
\]

\[
\text{Chl.’b’ mg/g fresh weight of the tissue} = \frac{22.9 \times (\text{O.D at 660}) - 4.68 \times (\text{O.D at 600})}{1000 \times W} \times V
\]

\[
\text{Total chl. mg/g fresh weight of the tissue} = \frac{\text{O.D at 660} \times 1000 \times V}{34.5 \times 1000 \times W}
\]

Where,

- O.D = Optical density at 600 and 660 nm
- V = Volume of extract in milliliters
- W = Fresh weight of the sample in gram
Enzymes

Estimation of $\alpha$-amylase activity (mg of glucose/mg of protein)

Sample preparation

One gram fresh weight of 15$^{th}$ day old seedlings were homogenized in 10ml of ice cold 10mM Calcium Chloride and kept overnight at 4°C. Later homogenate were centrifuged at 20,000 rpm for 20 minutes. The supernatant was used for the enzyme assay by the method suggested by Bernfield (1955). The reducing sugar released by action of enzyme on soluble starch was used to estimate $\alpha$-amylase activity.

Estimation of Activity

One ml of enzyme extract was taken in a test tube; one ml of 1% soluble starch was added and incubated at 37°C for 15 minutes, with occasional stirring. The enzyme activity was stopped by adding 2ml of 3,5 Dinitrosalicylic acid (DNS) reagent. The content was heated for five minutes in boiling water bath, one ml of 40% Rochelle salt was added, when the contents in the tube was still warm. Then tubes were cooled under tap water and diluted to 10ml with distilled water. Sample blank was prepared by adding DNS before incubation. The colour developed was read at 540nm in a spectrophotometer against the reagent blank. A unit of $\alpha$ – amylase was expressed as mg of maltose produced during 5 min incubation with 1% Starch and the specific activity was expressed in terms of mg of glucose per mg of protein.
Catalase Activity (units/gram weight of tissue)

The activity of catalase was assayed by the Loggini method (1990). Catalase enzyme catalyses the decomposition of H$_2$O$_2$. The principle of determining the catalytic property of catalase is based upon the rate of absorption. On decomposition of H$_2$O$_2$ by catalase the absorption decreases with time. The enzyme activity could be arrived at from this decrease.

Sample Preparation

One gm of fresh sample was homogenized using (0.1M) phosphate buffer (pH-6.8) in a mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 20min at 1-4$^\circ$C and the supernatants were taken for enzyme assay.

Estimation of Catalase activity: 3ml of H$_2$O$_2$–PO$_4$ was taken in a cuvette and 0.01ml of enzyme extract was added. The time required to change in absorbance from the 0.45 to 0.4 was recorded at 240nm using UV spectrophotometer.
Fig. 4.3: Rice Cultivar B.R.2655 grown in field
Fig. 4.3a: Rice Cultivar B.R.2655 grown in pot
Fig. 4.4: Rice Cultivar IET-7575 grown in field
Fig. 4.4a: Rice Cultivar IET-7575 grown in pot
Fig. 4.5: Rice Cultivar IR 30864 grown in field
Fig. 4.5a : Rice Cultivar IR 30864 grown in pot
Fig. 4.6: Rice Cultivar KMR-3R 3R grown in field
Fig. 4.6a: Rice Cultivar KMR-3R 3R grown in pot
Fig. 4.7: Rice Cultivar KRH – 2 grown in field
Fig. 4.7a: Rice Cultivar KRH – 2 grown in pot
Fig. 4.8: Rice Cultivar MTU 1001 grown in field
Fig. 4.8a: Rice Cultivar MTU 1001 grown in pot
Fig. 4.9: Rice Cultivar MTU 1010 grown in field
Fig. 4.9a: Rice Cultivar MTU 1010 grown in pot
Fig. 4.10: Rice Cultivar THANU grown in field
Fig. 4.10a: Rice Cultivar THANU grown in pot
Physiological Parameters

Plants were carefully removed from the pots and field after 60 days of transplantation so as to obtain plants with intact roots. The roots were carefully washed in water to remove soil particles attached to it. The root portion was separated from the shoot. After separation they were cut into small pieces, dried in sunlight for 10 days. They were further dried in a hot air oven at 70°C for eight hours. The dried plant parts were powdered in a pestle and mortar. The samples were preserved for future analysis. The methods of germination, transplanting and removal are same as described by Nene and Thapliyal (1971).

The digestion of the plant material and analysis of the samples to find out the nutrient uptake was carried out following the procedures given in the Laboratory Manual for physiological studies of rice by Shouichi Yoshida et al., (1972).

Sample Preparation

Sample extract was prepared by placing 1gm of dried ground plant material into a 100ml Pyrex volumetric beaker. 10ml of di-acid mixture [nitric acid (HNO₃) and perchloric acid (HClO₄) (9:4 ratio)] was added and allowed to pre digest under a fume hood for atleast 2 hours. The mixture was heated gently over a hot plate so as to avoid excessive frothing. Sample was digested, on a hot plate until it reduces to its 50% of the content. Sample extract was cooled and filtered through an acid washed filter paper (Whatmann No.1) sample extract was made upto 50ml with distilled water.

Sample extract was used to estimate uptake of Potassium, Sodium and Calcium by using Flame Photometer. Analysis of Nitrogen by nitrate estimation and elements like Copper, Zinc, Iron and Manganese were determined using Atomic Absorption Spectrophotometer. Phosphorus estimation was done by calorimetric method.
Nitrogen Estimation in Nitrate form by Brucine Sulphate Method (mg/gram fresh weight of tissue)

10ml of digested samples extract were taken in test tubes. All the test tubes were placed in a wire rack. The rack was placed in cool water bath and 2ml of NaCl solution was added. About 10ml of concentrated Sulphuric acid (H₂SO₄) was added and contents were mixed thoroughly. Then 0.5ml of Brucine reagent was added. The test tube rack was placed in hot water bath for 20min. Contents were cooled and readings were taken at 410nm. Concentration of Nitrate Nitrogen (NO₃⁻ N) was calculated from standard graph. Standard graph was prepared using Potassium Nitrate (KNO₃), ranging concentration between 0.1 to 1.0 mg N/L.

Estimation of Phosphorus in Sample by Colorimetric Method (mg/gram fresh weight of tissue)

The Phosphorus content in the sample was determined by colorimetric method. 1ml of the sample extract was placed into 50ml volumetric flask, 10ml of Ammonium Molybdate Vandate reagent was added to each flask and total volume was made with distilled water and properly shaken. The solutions were kept for 30 minutes for the development of proper colour. Then the reading was taken at 420 nm, against reagent blank. The standard graph was plotted using analytical grade Potassium di Hydrogen Phosphate (KH₂PO₄) and amount of Phosphorus was calculated (mg P/ml).

Estimation of Iron, Copper, Zinc and Manganese by Atomic Absorption Spectrophotometer Method (ppm/gram fresh weight of tissue)

Micro nutrient elements like Iron, Zinc, Copper and Manganese were estimated in the sample extract by using Atomic Absorption Spectrophotometer. One ml of the sample extract was used each time for estimation. Samples were fed to Atomic Absorption Spectrophotometer and readings were recorded. Each time instrument was standardized with particular standard solutions of Iron, Zinc, Copper and Manganese.
Preparation of Standard Solutions

Copper, Zinc, Iron stock solutions containing 1000ppm of each elements were purchased from E. Merck, (India) Limited, Worli, Mumbai from which working standard solutions were prepared.

Iron Standard solutions

10ml of stock solution was taken in 100ml volumetric flask and made upto 100ml with distilled water to prepare working standard solution (100 ppm).

Instrument Working Standard Solution

10ml aliquot of working standard solution was taken and made up to 100ml with distilled water which contained 10ppm Iron. An Iron standard ranging from 2ppm, 4ppm and 6ppm were prepared by placing 2 ml, 4ml, and 6ml of 10ppm Iron solution and was made upto the mark with distilled water. These standards were used to calibrate the instrument.

Zinc Standard Solutions

10ml of stock solution was made upto 100ml by adding distilled water in a volumetric flask. This solution contains 100ppm (working standard solution).

Instrument Working Standard Solutions

10ml aliquot of working standard solution was taken and made upto 100ml with distilled water which contained 10ppm of Zinc. A Zinc standard ranging from 0.5ppm, 1ppm and 2ppm were prepared by placing 0.5ml, 1ml and 2ml of 10ppm Zinc solution and was made upto 100ml. These standards were used to calibrate the instrument.

Copper Standard Solutions

10ml of stock solution was placed in 100ml volumetric flask and made upto the mark with distilled water which contains 100ppm of Copper. This was used as working standard solution.
Instrument Working Standard Solutions

10ml aliquot of working standard solution was placed in volumetric flask and made upto 100ml with distilled water which consists of 10ppm of Copper. Copper Standards ranging from 1ppm, 2ppm and 5ppm were prepared by placing 1ml, 2ml and 5ml of 10ppm Copper solution and was made upto the mark with distilled water. These standards were used to calibrate the instrument.

Manganese Standard Solutions

Stock solution: 3.0764 gram of AR grade Manganese sulphate (MnSO₄) was dissolved in 1000ml of distilled water. One litre of this solution contains 1000ppm of Manganese. 10ml of stock solution was made upto 100ml with distilled water in a volumetric flask. This solution contains 100ppm of Manganese. It was used as working standard solution.

Instrument Working Standard Solution

10ml of working standard solution was placed in 100ml volumetric flask and made upto the mark with distilled water, which contains about 10ppm of Manganese. Manganese standards ranging from 2ppm, 4ppm, and 6ppm were prepared by placing 2ml, 4ml, and 6ml of the 10ppm manganese solution and was made upto 100ml with distilled water. These standards were used to calibrate the instrument before estimation.

Estimation of Potassium, Calcium and Sodium by Flame Photometer Method (ppm/gram fresh weight of tissue)

Estimation of Potassium, Calcium and Sodium was done by using Flame Photometer.
Standard solutions of Potassium, Sodium and Calcium

1000ppm stock solution of Potassium was prepared by dissolving 1.907g of dried AR grade Potassium Chloride (KCl) in 1 litre of de-ionised distilled water. Potassium standards like 0ppm, 20ppm, 40ppm, 60ppm, 80ppm, and 100ppm were prepared by placing 0ml, 2ml, 4ml, 6ml, 8ml and 10ml of 1000ppm Potassium solution respectively in separate 100ml volumetric flasks and final volume was made to 100ml with 1N HCl.

1000ppm stock solution of Sodium was prepared by dissolving 2.542 of dried AR grade Sodium Chloride (NaCl) in 1 litre of de-ionised distilled water. Then Sodium standards like 0ppm, 5ppm, 10ppm, 20ppm and 40ppm were prepared by placing 0ml, 0.5ml, 1ml, 2ml and 4ml of 1000ppm Sodium solutions respectively in 100ml of volumetric flasks and the final volume was made to 100ml with 1N HCl.

1000 ppm stock solution of Calcium was prepared by dissolving 2.497g of dried AR grade Calcium Carbonate (CaCO₃) in 50ml of 1N HCl in a 100ml volumetric flask. Solution is gently boiled for 3min and allowed to cool. Then solution was transferred to 1 litre volumetric flask and made upto the mark with de-ionised distilled water. Then Calcium standards ranging from 20ppm, 40ppm, 60ppm, 80ppm, 100ppm, 120ppm and 140ppm were prepared by placing 2ml, 4ml, 6ml, 8ml, 10ml, 12ml and 14ml of 1000ppm Calcium solutions respectively in 100ml of volumetric flask and the final volume was made upto 100ml with 1N HCl.
Gas Exchange Parameters

Various gas exchange traits such as net photosynthetic rate (A), intercellular CO₂ concentration (Ci) and stomatal conductance (gs) was measured using portable photosynthetic system. In this investigation, Portable Photosynthetic System LICOR, 6400 (Infra-Red Gas Analyser) was used to measure gas exchange parameters.

Fig. 4.11 : Portable Photosynthetic System LICOR-6400 (Infra-Red Gas Analyser)
Fig 4.12: Photos on measurement of Net photosynthetic rate, Stomatal conductance and intercellular carbon dioxide concentration using Portable Photosynthetic System LICOR-6400 in field
The gas exchange parameters were determined in the field condition. 60 days old rice plant leaves were used to determine the parameters before removal of plants for estimation of nutrient uptake. The 3rd top fully expanded leaf from the apex was clamped to the leaf chamber and the observations were recorded when A, gs and Ci reached a stable value, all gas exchange parameters were recorded between 9am and 12noon on bright sunny day.
Determination of Carbon Isotope Discrimination

Measuring carbon isotope discrimination by IRMS method (Farquhar et al., 1989)

Preparation of Samples

The leaf samples were oven dried at $80^\circ$C for three days and finely powdered using a ball mill. About one mg of dry leaf powder was taken in silver capsules to determine the carbon isotope using an IRMS at the National facility for stable isotope studies in biological sciences, UAS, Bangalore.

Fig 4.13: Schematic representation of the Flash elemental analyzer and the combustion and reduction of the organic sample

Finely powdered leaf samples were accurately weighed in the range of 0.8 to 1.0 mg into silver capsules. The crimped capsules with the sample were placed sequentially in the carousel of the auto-sampler. The samples are dropped at precise times along with an injection of pure O$_2$ into the oxidation reaction.

The combustion (oxidation) reactor consisted of chromium oxide and silvered cobaltous-cobaltic-oxide in a quartz column heated to 1050$^\circ$C. The biomass is completely oxidized to produce CO$_2$, N$_2$O and H$_2$O. These gases were swept into the
reduction furnace using helium carrier gas (purity 99.995%). The reduction furnace consisted of reduced Copper in quartz tubes heated to 680°C. In this reactor, the N₂O is reduced to N₂ and the excess O₂ is absorbed. The resultant gases are then flushed through scrubbers to trap water. The CO₂ and N₂ gases are then passed through a GC column (5 Å molecular sieve) to effectively separate these gases before introduction into the ion source of the IRMS. At the ion source, CO₂ was ionized by electron impacts ionization to produce molecular radicals. These CO₂ radicals are accelerated under the influence of high voltage potential of 3000 volts through a strong magnetic field. When accelerated radicals pass through a strong magnetic field, the radicals are deflected with the radius of deflection being proportional to the molecular mass of the radicals. These deflecting ¹²CO₂ and ¹³CO₂ were collected by Faraday cups and the amplified signals were transmitted to the computer. Thus

\[ \Delta^{13}C \text{ is computed from the following equation.} \]

\[ \Delta^{13}C \text{ (‰) = } \frac{\delta^{13}C_a - \delta^{13}C_p}{1 + \delta^{13}C_p/1000} \]

Where, \( \delta^{13}C_a \) is the isotopic composition of the ambient air corrected against the PDB standard. In an unpolluted air, the \( \delta^{13}C_a \) is considered as \(-8\%\). \( \delta^{13}C_p \) is the isotopic composition of the organic sample corrected against the PDB standard as determined by the IRMS.

**Statistical Analysis**

The data recorded were subjected to,

One – Way ANOVA post hoc non parametric SNK test,

‘t’ test significance through graph pad software on line analysis and

“Nestedness Pattern” analysis, to arrive at precise conclusions.