3. MATERIALS AND METHODS

3.1. Physicochemical Analysis

All the parameters in physicochemical analysis of the effluent were done as per the methods of APHA, AWWA and WPCF, (1992) and Abbasi, (1998).

3.1.1. Colour

Colour was recorded based on visual observation.

3.1.2. pH

pH was measured using a pre-calibrated pH meter with an accuracy of ± 0.05. 50ml of the effluent was taken in a clean and dry borosilicate beaker and the electrode was immersed in it so that the value was displayed.

3.1.3. Apparent Specific gravity

Apparent Specific gravity was estimated by weighing method using a 50ml weighing bottle.

3.1.4. Electrical Conductivity

Electrical conductivity was measured using a conductivity meter.

3.1.5. Total Dissolved Solids and Suspended Solids

These were determined by the gravimetric method

3.1.6. Chemical Oxygen Demand (COD)

COD was determined by refluxing the sample with standard potassium dichromate and sulphuric acid and titrating against standard ferrous ammonium sulphate.

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3.1.7. **Biochemical / Biological Oxygen Demand (BOD)**

The 5-day BOD test method was adopted and the samples were incubated at 20°C for five days in overflowing air tight bottles. Dissolved oxygen was measured initially and after incubation and the BOD values were calculated.

3.1.8. **Total Alkalinity**

Alkalinity was measured by titrating the sample with standard 0.02N sulphuric acid and expressed in terms of calcium carbonate.

3.1.9. **Total volatile acidity**

This was estimated by titrating the samples with 0.02N sodium hydroxide solution using methyl orange indicator.

3.1.10. **Chloride**

Chloride content was estimated through volumetric titration of sample against standard silver nitrate using potassium chromate as indicator.

3.1.11. **Sulphate**

Sulphate content was measured turbidimetrically at 420 nm, where the turbidity was formed of barium sulphate suspension.

3.1.12. **Nitrogen**

Nitrogen content was determined by using Kel Plus distillation apparatus (modified Kjeldahl apparatus) after digestion of the samples (Jackson, 1958).

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Studies on the effect of distillery effluent on rice (*Oryza sativa* L.)
3.1.13. **Phosphorus**

Phosphorus content in the sample was estimated by molybdenum blue method using a spectrophotometer at 660nm.

3.1.14. **Potassium**

Potassium content was estimated by flame photometric method by using direct reading type flame photometer (Jackson, 1958).

3.1.15. **Phenolics**

The sample was distilled and the collected distillate was reacted with 4-aminoantipyrine and potassium ferricyanide. The phenolic concentration of the sample was estimated from standard calibration curve.

3.1.16. **Oil and Grease**

Soxlet extraction method was followed to estimate the presence of oils and grease in the sample.

3.2. **Materials**

Certified seeds of two rice varieties, MO-16 (Uma) and MO-19 (Krishnanjana), from Regional Rice Research Station, Kerala Agricultural University, Mancompu, were used as the seed source for conducting the experiment as these two varieties are locally cultivated in most of the rice fields of kuttanad.

The seeds were washed in 0.05% mercuric chloride mixed with a dilute detergent to provide surface sterilization and were used for the experiment.

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Distillery effluent was collected from the outlet of M/s McDowell’s & Co. Varanad, Cherthala, Alappuzha, Kerala. Effluent required for the experiments was collected just before the use in a sterilized glass container which was acid washed and rinsed in distilled water. Different concentrations of the effluent were prepared (1, 5, 10, 20, 30, 40 & 50%) using distilled water/irrigation water.

3.3. Germination Studies

For germination studies, 25 seeds were sown evenly in pre-sterilized petriplates of 11cm diameter lined with a layer of Whatman No: 1 filter paper. To each petriplate, 10ml of different concentrations (1, 5, 10, 20, 30, 40 & 50%) of distillery effluent was added. A control was also maintained and for this, double distilled water was used instead of the effluent. Three replications were maintained for each treatment.

The germination study was carried upto 96 hours and after every 24 hours the filter paper and the solutions were changed to avoid contamination and inhibition of germination and growth by the leachates.

3.3.1. Germination Percentage

Number of germinated seeds was recorded at every 24 hours upto 96 hours of sowing. The criterion followed for the germination was the emergence of the radicle and the viability was
expressed as the germination percentage and was calculated using the following formula.

\[
\text{germination percentage} = \frac{\text{No. of seeds germinated}}{\text{Total No. of seeds sown}} \times 100
\]

3.3.2. Mobilization Efficiency Percentage

The Mobilization Efficiency Percentage for the food reserve was calculated using the following formula (Doijode and Raturi, 1990).

\[
\text{Mobilization Efficiency Percentage} = \frac{\text{Dry weight of seedling}}{\text{Dry weight of seed}} \times 100
\]

3.3.3. Seedling growth

Growth measurements were taken on seedlings after 96 hours of sowing. Three seedlings from each replication were randomly selected and growth parameters like root length, shoot length, fresh weight, dry weight were measured and shoot root ratio (on length basis and dry weight basis) and vigour index (length basis and dry weight basis) were calculated.

3.3.3.1. Root length

Root length was measured using a thread and a metre scale and the mean length was calculated and expressed in cm.

3.3.3.2. Shoot length

Shoot length was measured from the collar region to the tip region of the shoot. The mean shoot length was calculated and expressed in cm.

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3.3.3.3. Seedling Fresh weight

The fresh weight of the root and shoot was weighed separately in an electronic balance (Sartorius) and the mean values were expressed together as seedling fresh weight in mg.

3.3.3.4. Dry Matter Production Percentage

For calculating the dry weight, the shoot and root were kept separately in labeled packets in a hot air oven at 80°C till it attains a constant weight. The dry weight was weighed using Sartorius electronic balance. The percentage dry matter production of the seedling was calculated using the formula.

\[
\text{Dry matter production percentage} = \frac{\text{Average dry weight}}{\text{Average fresh weight}} \times 100
\]

3.3.3.5. Shoot Root Ratio (length basis)

Shoot Root Ratio on length basis was calculated using the following formula

\[
\text{Shoot Root Ratio (length basis)} = \frac{\text{Mean length of shoot}}{\text{Mean length of root}}
\]

3.3.3.6. Shoot Root Ratio (dry weight basis)

Shoot Root Ratio on dry weight basis was calculated using the following formula

\[
\text{Shoot Root Ratio (dry weight basis)} = \frac{\text{Mean dry weight of shoot}}{\text{Mean dry weight of root}}
\]
3.3.3.7. Seedling Vigour Index (SVI)

Seedling Vigour Index on length basis was calculated by multiplying the germination percentage with the mean seedling length (Abdul Baki and Anderson, 1973) and on dry weight basis by multiplying the germination percentage with the mean dry weight of seedlings (Ching, 1973).

Seedling Vigour Index (length basis)  
\[ = \% \text{ germination} \times \text{seedling length} \]

Seedling Vigour Index (dry weight basis)  
\[ = \% \text{ germination} \times \text{seedling dry weight} \]

3.5. Field Studies

Field trial was conducted on a farmer’s field (private holding) at Punnapra Village in Ambalapuzha Taluk, Alappuzha District. This region is included in the Kuttanad area, (below sea level) one of the important area where there is massive rice cultivation.

Land preparation was done according to the standard procedure (Jose 2002). The seeds of MO 16 (Uma) and MO 19 (Krishnanjana) were given wet seed treatment. Seeds were soaked in 0.2% Bavistin solution for 15 minutes and then in distilled water for 12 hours. The solution was drained and the seeds were kept for sprouting in a moist gunny bag. After 72 hours, the germinated seeds were sown in seed beds raised in the nursery. These were allowed to grow for 20 days with periodical watering. Healthy seedlings were selected from the stand after 20 days and were transplanted to the plots.
The experimental field was ploughed and the weeds were removed. 2m x 2m plots were laid in a Randomised Block Design (RBD) with a bund of 30cm between each plot. A thin film of water was maintained in each plot and the excess water was drained out. The seedlings selected from the nursery beds were transplanted singly in rows with a gap of 10cm between plants and 20cm between rows. Plants were allowed to establish in the plots and causalities were replaced within 4 days.

After 10 days of transplanting, 10 litres of different concentrations of the effluent (1, 5, 10, 20, 30, 40 & 50 %) were irrigated to the respective plots. The control plots were irrigated with the irrigation water. On every 15th day, irrigation with different concentrations of the distillery effluent was repeated. Weeds were removed then and there. Pest and disease management was also done according to the recommended package of practice for rice crop (Jose, 2002). Three replicates were maintained for each treatment and the experiment was repeated twice.

Three plants were randomly selected for the growth measurements from each replication of the two varieties. These plants were uprooted on 30th, 60th and 90th day after transplanting (DAT). Much care was taken to ensure that no damage was caused to the root system at the time of uprooting. The plants were collected in the early hours of morning before 8a.m so that the

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metabolic fluctuations due to photosynthetic activity could be minimized. The plant samples were wrapped in butter paper before bringing to the laboratory to prevent drying. The plants were washed thoroughly in water to remove the soil particles and were pressed gently between the blotting sheets to remove the excess water present on the plant surface.

Growth parameters such as root length, shoot length, number of tillers, fresh weight and dry weight of root and shoot and leaf area were measured. From these measurements, percentage dry matter production, shoot root ratio on length basis and dry weight basis, relative growth rate, net primary production, net assimilation rate and leaf area ratio were calculated.

3.5.1. Growth attributes
3.5.1.1. Root length
The length of the root was measured from the base of the stem to the longest root using a meter scale and the mean root length was calculated and expressed in cm.

3.5.1.2. Shoot length
The length of the shoot was measured from the base of the stem to the tip of the leaf using a meter scale and the mean shoot length was calculated and expressed in cm.

3.5.1.3. Number of tillers
The total number of tillers produced at different stages of growth was counted and the mean number expressed as the number of tillers.
3.5.1.4. **Fresh weight**

The fresh weight of root and shoot were weighed separately using Sartorius electronic balance, pooled together and the mean was expressed in gram.

3.5.1.6. **Percentage Dry Matter Production**

After taking the fresh weight, the root and shoot samples of each treatment were kept separately in labeled packets in a hot air oven at 80 ± 1°C for a period of one week for complete and uniform drying. After uniform drying, the dry weight was measured and the percentage dry matter production was calculated using the formula

\[
\text{Percentage Dry matter production} = \frac{\text{Dry weight}}{\text{Fresh weight}} \times 100
\]

3.5.1.10. **Leaf Area**

Leaf area was calculated based on the method proposed by Reddy *et al.*, (1999). For this a linear regression equation was formulated using randomly chosen 20 leaves. The equation was fit for each variety using the length (l) and maximum width (b). The values were expressed in cm².

- for MO 16 \( y = 0.687 (l \times b) + 0.584 \)
- for MO19 \( y = 0.535 (l \times b) - 0.362 \)

3.5.1.4. **Shoot Root Ratio (length basis)**

Shoot root ratio on length basis was calculated using the formula

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Studies on the effect of distillery effluent on rice (*Oryza sativa* L.)
Shoot root ratio (length) = \( \frac{\text{Mean length of shoot}}{\text{Mean length of root}} \)

### 3.5.1.7. Shoot Root Ratio (dry weight basis)

Shoot root ratio on dry weight basis was calculated using the formula

\[
\text{Shoot root ratio (dry weight)} = \frac{\text{Mean dry weight of shoot}}{\text{Mean dry weight of root}}
\]

### 3.5.1.8. Relative Growth Rate

Relative growth rate was calculated using the formula of Williams (1946) and Srinivasulu et al., (1999) and the values were expressed in g g\(^{-1}\) day\(^{-1}\).

\[
\text{Relative Growth Rate} = \frac{\log_e W_2 - \log_e W_1}{t_2 - t_1}
\]

- \(W_2\) - final weight, \(W_1\) - initial weight, \(t_2\) - final time, \(t_1\) - initial time

### 3.5.1.9. Net Primary Production

Net primary production was calculated using the formula of Banergee et al., (2004) and the means were expressed in g plant\(^{-1}\) day\(^{-1}\).

\[
\text{Net primary production} = \frac{W_2 - W_1}{t_2 - t_1}
\]

- \(W_2\) - final weight, \(W_1\) - initial weight, \(t_2\) - final time, \(t_1\) - initial time

### 3.5.1.9. Net Assimilation Rate

Net assimilation rate was calculated using the formula of Noggle and Fritz (2002) and the means were expressed in g cm\(^{-2}\) day\(^{-1}\).
Net assimilation rate = $\frac{W_2 - W_1 \times \log_e \frac{LA_2}{LA_1}}{t_2 - t_1}$

$W_2$ - final weight, $W_1$ - initial weight, $t_2$ - final time, $t_1$ - initial time, $LA_2$ - leaf area at $t_2$, $LA_1$ - leaf area at $t_1$

3.5.1.11. **Leaf Area Ratio**

Leaf area ratio was calculated by employing the formula of Radford (1967) and the values are expressed in cm$^2$ g$^{-1}$.

Leaf Area Ratio = \(\frac{\text{Leaf area per plant}}{\text{Plant dry weight}}\)

3.5.2. **Yield attributes**

To make an assessment on the effect of different concentrations of the effluent on various yield components, the following parameters were studied and the methods of measurement are described below.

3.5.2.1. **Productive Tillers**

The tillers bearing the ear heads were collected and expressed as number of productive tillers.

3.5.2.2. **Panicle Length**

The ear heads were collected and the length of the panicle was measured and the mean values expressed in cm.

3.5.2.3. **Panicle Weight**

The collected panicles were weighed using Sartorius electronic balance and the means were expressed in grams.

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3.5.2.4. Thousand Grain Weight

Grains were collected and pooled and the weight of 100 seeds was weighed using Sartorius electronic balance. From this 1000 grain weight was calculated and expressed in grams.

3.6. Metabolic Drift during Different Stages of Growth

Biochemical analysis was done on the germinated seedlings (after 96 hours of sowing) and flag leaves from the vegetative phase (at the tillering stage) and reproductive phase (at seed setting stage) from randomly selected plants. The methods employed in the analysis are presented below.

3.6.1. Metabolites

3.6.1.2. Total Sugar Content

Total sugar content was estimated by employing Nelson Somogyi method (Warten and Mc Carty, 1972). 100mg of the samples were homogenized and boiled in 80% ethanol, centrifuged and the supernatant was collected and made up to a known volume using distilled water. To 5ml of the extract 3ml of 1N HCl was added and kept in boiling water bath for 20 minutes to hydrolyze non-reducing sugars. It was cooled and neutralized by adding 3ml of 1N NaOH solution. To the mixture, 1ml of 25% lead acetate solution and 1ml of 25% sodium carbonate solution were added, made up the volume to 15ml by adding distilled water. It was filtered and from this 1ml of the filtrate (aliquot) was mixed with 1ml of Nelson Somogyi reagent and the test tube was closed
and heated at 100°C in a water bath for 20 minutes. It was then cooled rapidly and 1ml of arsenomolybdate was added and mixed thoroughly by shaking the tube for 5 minutes. The final volume of the resultant bluish green solution was made upto 5ml with distilled water and the absorbance was recorded at 540nm. The following regression equation was prepared by taking different concentrations of glucose as the standard and was expressed in µg mg⁻¹ fresh weight.

\[ x = 234.59 y - 4.28 \]  
(y is the absorbance of the sample)

### 3.6.1.1. Reducing Sugar Content

Reducing sugar content was estimated by employing Nelson Somogyi method (Warten and Mc Carty, 1972). 100mg of the samples were homogenized and boiled in 80% ethanol and centrifuged. The supernatant was made up to 10ml with distilled water. From this, 5ml was taken in a test tube and added 1ml of 25% lead acetate solution followed by 1ml of 25% sodium carbonate solution. The volume was made up to 10ml with distilled water and filtered and the filtrate was used as the aliquot. The content of sugar was estimated by employing Nelson-Somogyi method as described in 3.5.1.1. for total sugar and expressed in µg mg⁻¹ fresh weight.

### 3.6.1.3. Starch Content

The starch content was estimated as per the method of Manickam and Sadasivam (1996). 100mg of the samples were
homogenized in hot 80% ethanol to remove sugars and the residue was retained and dried over a water bath. To the residue added 6.5ml 52% perchloric acid and 5ml distilled water. This was incubated at 0°C for 20 minutes and the supernatant was extracted and made up to a known volume. 0.2ml aliquot was pipetted out and added 0.8 ml distilled water and 4ml of anthrone reagent. The mixture was boiled for 8 minutes in a water bath and cooled in ice bath subsequently. The colour intensity was measured at 630nm in the visible range spectrophotometer. A regression equation was formulated and expressed in $\mu g \text{ mg}^{-1}$ fresh weight.

$$x = 347.22y + 1.39 \text{ (y is the absorbance of the sample)}$$

3.6.1.4. Protein Content

Protein content was estimated as per the method of Lowry et al., (1951). 100mg of the samples were crushed in 80% ethanol and centrifuged. The residue was washed first in 5% perchloric acid (to remove sugars and soluble nitrogen fractions) and secondly in a mixture of ethanol: ether: chloroform (2:1:1) to remove soluble fractions and lipids. Then the residue was suspended in 1M trichloroacetic acid (cold) centrifuged and the residue was dissolved in 1N NaOH and kept for one hour at room temperature. After centrifuging the mixture, the supernatant was collected and made up to a known volume and used as aliquot. To a known volume (2ml) of aliquot 5ml of Lowry reagent C prepared by mixing 50ml of Lowry reagent A and 1 ml of Lowry reagent B was

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added and incubated for 10 minutes at room temperature. In control sets the aliquot was replaced by distilled water. After 10 minutes 0.5ml folin phenol reagent was added to the mixture and incubated for 30 minutes at room temperature. The characteristic blue colour indicated the presence of protein. The absorbance of this solution was read at 660nm on a spectrophotometer. Optical density was calculated by using the regression formula prepared from Bovine serum albumin as standard protein and the results are expressed in $\mu$g mg$^{-1}$ fresh weight.

$$x = 595.31y - 25.62$$ (y is the absorbance of the sample)

### 3.6.1.5. Total Free Amino Acid Content

Total free amino acid content was estimated as per the method of Lee and Takahashi (1966). 100mg of the samples were crushed in 80% ethanol and centrifuged. To a known volume of the extract, 5ml Nihydrin reagent was added and shaken vigorously and kept in boiling water bath for 12 minutes. The tubes were cooled to room temperature under running tap water and the absorbance was read at 570nm on a visible range spectrophotometer. Amino acid content was estimated using the following regression equation prepared using glycine as standard and the results are expressed in $\mu$g mg$^{-1}$ fresh weight.

$$x = 127.7y + 7.36$$ (y is the absorbance of the sample)
3.6.1.6. Proline content

Proline content was estimated as per the method of Bates *et al.*, (1973). 100mg of the samples were homogenized and extracted in 3% sulphosalicylic acid. The supernatant was separated by centrifugation and was made up to a known volume. 2ml of this aliquot was added to 2ml acid ninhydrin (2%) and 2ml glacial acetic acid and kept in boiling water bath for 1 hour. The reaction was terminated in ice bath. The mixture is then treated with 6ml toluene and mixed well for 30 seconds. The toluene layer was separated using a separating funnel and brought to room temperature. The red colour intensity was measured at 520nm on a visible range spectrophotometer. The proline content was calculated using the regression equation formulated using standard proline and expressed in µg mg\(^{-1}\) fresh weight.

\[ x = 1116.70y + 0.72 \] (y is the absorbance of the sample)

3.6.1.7. Total Phenol content

The total phenol content was estimated as per the method of Farks & Kirly (1962). 100mg of the samples were homogenized in 80% ethanol and the supernatant was collected by centrifugation. From this, 1ml of aliquot was taken and mixed with 2ml of 20% sodium carbonate and 1ml of folin phenol reagent. This was kept in boiling water bath for 10 minutes till the blue colour developed. It was cooled and diluted to a known volume and the absorbance was

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measured at 660nm. A regression equation was formulated using gallic acid as standard and expressed in \( \mu g \text{ mg}^{-1} \) fresh weight.

\[ x = 96.05y + 10.3 \quad (y \text{ is the absorbance of the sample}) \]

### 3.6.2. Enzyme Activity

#### 3.6.2.1. Hydrolyzing Enzymes

##### 3.6.2.1.1. Amylase Activity

Specific activity of the enzyme amylase was estimated as per the method of Sadasivam and Manickam (1996). 100mg of the samples were ground in 10mM ice-cold calcium chloride and incubated for three hours at room temperature. The extract was then centrifuged at 54000g at 4°C for 20 minutes. To 1ml supernatant, added 1ml 1% starch solution and incubated at room temperature for 15 minutes. To this added 2ml DNS reagent and kept in boiling water bath for 5 minutes. To the warm tubes, added 1ml of 40% potassium sodium tartarate solution. In control tube the reaction was terminated at 0 time. This was then made up to 10ml volume by adding distilled water. The absorbance was read at 560nm. A regression equation was formulated using maltose as standard. This is expressed as \( \mu g \) maltose produced min\(^{-1}\) mg\(^{-1}\) fresh weight with 1% starch.

\[ x = 3.636y - 0.0011 \quad (y \text{ is the absorbance of the sample}) \]

##### 3.6.2.1.2. Invertase Activity

Specific activity of the enzyme invertase was estimated as per the method of Sasidharan, (1993) (dinitrosalicylic acid
method). This enzyme breaks down sucrose to fructose and glucose. 100mg of the samples were homogenized in 3ml of 0.1M phosphate buffer (pH 7), centrifuged, and the supernatant was used as the enzyme source. 2.5ml of 0.3M sucrose solution was mixed with 1.5ml of 0.2M citrate phosphate buffer (pH 4.5) in the test tube, 1ml of the enzyme source (extract) was added to this and the mixture was incubated at room temperature for 15 minutes. The reaction was stopped by adding 0.5ml of 1% sodium hydroxide. The solution was centrifuged at 2000g for 10 minutes and estimated the glucose/fructose in the supernatant.

For the estimation of the specific activity, measured out a small aliquot (0.2ml) from the supernatant and added distilled water and made up to 3ml. To this solution, 3ml of DNS reagent was added and transferred to a boiling water bath for 5 minutes. When the contents of the tubes were warm, 1ml of 40% Rochelle salt (Potassium Sodium Tartarate) solution was added and the mixture was cooled and the absorbance was read at 525nm. Invertase activity was expressed as the µg glucose produced min⁻¹ mg⁻¹ fresh weight at a specific temperature. The calculation was done using the regression equation prepared with standard glucose.

\[ x = 234.59y - 4.28 \] (y is the absorbance of the sample)
3.6.2.1.3. **Protease activity**

Specific activity of the enzyme protease was estimated as per the method of Dubey (1982). 100mg of the fresh samples were homogenized and extracted in 1ml 0.1M phosphate buffer (pH 7) and used as the enzyme solution. 1% Casein solution prepared in 0.1M phosphate buffer was used as the substrate for the estimation of protease activity. 0.1ml substrate solution and 0.2ml enzyme solution were mixed in a test tube. A control was also maintained without the enzyme. The reaction mixture was incubated for one hour in a water bath at 37°C. Then 0.6ml 5% TCA was added to arrest the reaction and was kept for half an hour. The mixture was centrifuged at 10,000g for 10 minutes. The TCA soluble peptide fragments were measured at 660nm by the method of Lowry et al., (1951). One unit of protease is expressed as µg peptide fragments formed hour⁻¹ mg⁻¹ fresh weight. The calculation was done using the regression equation.

\[ x = 224.07y - 0.40 \]  
(y is the absorbance of the sample)

3.6.2.2. **Oxidizing Enzymes**

3.6.2.2.1. **Catalase activity**

Specific activity of the enzyme catalase was estimated as per the method of Sadasivam and Manickam (1996). 100mg of the fresh samples were weighed out and homogenized using a pre-cooled mortar and pestle and extracted with 2ml M/150 phosphate buffer (pH 7) at 4°C and spun at 15000g and the supernatant was
used for the assay. The reaction mixture was prepared by mixing 3ml H$_2$O$_2$-phosphate buffer and 0.04ml sample and mixed. The absorbance was measured using UV-Visible spectrophotometer at 240nm. The change in time was recorded for the decrease in absorbance from 0.45 to 0.4 by using a stopwatch. A control was also maintained with the sample mixed with H$_2$O$_2$ free phosphate buffer. The calculation was done as follows and expressed as µmoles of H$_2$O$_2$ split min$^{-1}$ mg$^{-1}$ fresh weight.

Catalase activity = $17 \times \frac{\text{Total volume of enzyme (extract volume)}}{\Delta t} \times \frac{\text{Volume of enzyme solution}}{x} \times \text{sample weight}$

3.6.2.2. Peroxidase activity

Specific activity of the enzyme peroxidase was estimated as per the method of Goliber (1989). 100 mg of the fresh samples were homogenized using a clean, dry, pre-cooled mortar and pestle in 1ml 0.1M phosphate buffer (pH 7). The extract was centrifuged at 15000g for 20 minutes at 4°C. The supernatant was collected and served as the enzyme source.

The reaction mixture was prepared by mixing 2ml 0.1M phosphate buffer (pH 7), 1ml 20mM guaiacol, 0.05ml 10mM H$_2$O$_2$ and 0.04ml enzyme solution, mixed well and noted the initial absorbance at 470nm against the blank. After incubating the mixture for 10 minutes the absorbance was recorded at 470nm and it is the final absorbance. Activity of the enzyme is expressed

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as difference in OD min\(^{-1}\) mg\(^{-1}\) fresh weight. The calculation is as follows.

\[
\text{Enzyme activity} = \frac{\text{Difference in OD} \times \text{Total volume of enzyme extract}}{\text{Volume of enzyme solution} \times \text{tissue weight} \times 10^{3}}
\]

### 3.4. Isozyme Studies by PAGE

#### 3.4.1. Preparation of Enzyme Extract

After 96 hours of sowing, 1g samples from each concentration was weighed and ground in liquid nitrogen and the enzymes were extracted in low temperature (4\(^{\circ}\)C) with 2ml of 0.05M Tris buffer, pH7.1. The crude extracts were then centrifuged at 12000g for 15 minutes at 4\(^{\circ}\)C and clear supernatants were collected. The duration of the extraction procedure was kept to the minimum to avoid denaturation of enzymes. The enzyme extracts were stored at –70\(^{\circ}\)C till used.

Concentration of protein in the enzyme extracts was estimated following the method of Lowry et al., (1951) using Bovine Serum Albumin as the standard. The final concentration of protein was equilibrated in all the samples by adding required volume of extraction buffer. Supernatants were mixed with 1M Sucrose (10\(\mu\)l), 0.1%(w/v) Bromophenol Blue (10\(\mu\)l) and vortexed for a few seconds before loading the extract on to the gel.

#### 3.4.2. Casting of Polyacrylamide Gels

##### 3.4.2.1. Separating gel

Two slab gel plates were assembled in a casting mode using 1.5mm thick spacers. Stock solutions were prepared as per

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standard protocols (Appendix-1). Required quantity of the stock solutions (Appendix-2) were mixed thoroughly except Ammonium Persulphate and TEMED. The mixture was degassed in a vacuum flask followed by the addition of Ammonium Persulphate and TEMED. The mixture was then gently swirled to mix. The gel solution after proper mixing was pipetted into the glass sandwich to a level of 4 centimeters from the top. A water layer of about 2mm thick was applied over the solutions before polymerization of the gel solution to make the gel surface uniform as evident from the sharp water gel interface visible after polymerization. After polymerization, water was poured off and 1ml of running gel overlay solution was applied on the gel surface.

3.4.2.2. Stacking gel

The preparation of stock solutions for stacking gel was detailed in (Appendix-1). Measured amount of stock solutions were mixed for stacking gels (Appendix-2). The mixture was degassed prior to the addition of Ammonium Persulphate and TEMED. The running gel overlay solution was poured off and stacking gel solution was gently poured into the sandwich over the polymerized separating gel. A comb was inserted into the sandwich to make wells in the stacking gel at regular intervals for the application of the extracts. The stacking gel was allowed to polymerize for half an hour.

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3.4.3. Electrophoresis of enzyme extract

Polyacrylamide gel electrophoresis of the enzyme extracts prepared from the treated seedlings was carried out using Hoeffer Vertical Slab Gel Electrophoresis System at 4°C. The polymerized gel was removed from casting mode having two spacers on two edges and lower free surface of separating gel, fitted with the electrophoresis apparatus. The comb was removed straight up from the stacking gel. Lower tank was filled with running buffer upto a certain mark in such a way that the lower free surface of separating gel remained immersed in the running buffer. If any air bubble gets trapped at the interface of lower free end of separating gel and lower tank buffer was coaxed away with a pipette.

The enzyme extracts were then underlaid in the wells of the stacking gel. Volume of each sample enzyme was adjusted in such a way that a fixed amount of the enzyme extract for electrophoresis for a particular enzyme system contained equal amount of proteins. Care was taken to avoid mixing of the samples with the reservoir buffer and also to avoid cross contamination of samples. Constant current applied for 1.5mm thick gel was 200V, initially it was 150V, but increased to 200V when dye front entered into the separating gel. The electrophoresis was done in cold chamber having constant temperature of 4°C, until the tracking dye had migrated to the bottom of the gel. With the completion of
the electrophoresis the gels were stained as per the staining protocol of the enzyme systems.

3.4.4. Staining method for Peroxidase

The staining ingredients like O-dianisidine and β-naphthol according to the required quantity (Appendix-4) were first dissolved in acetone and then 0.1M Tris-Acetate buffer was added to it. Volume was made up to 150ml with double distilled water. Mixture was filtered and just prior to use, the substrate, 3% hydrogen peroxide was added to it. The gels were incubated with staining mixture till reddish brown bands of peroxide appeared.

3.7. Photosynthetic Pigments

The chlorophyll content viz. chlorophyll-a, chlorophyll-b and total chlorophyll were estimated as per the method of Arnon (1949). 1gram of leaf samples from each concentration was weighed, cut into fine pieces and ground into fine pulp using a mortar and pestle with the addition of 20ml 80% acetone. This was then centrifuged at 5000 rpm for 5 minutes and the supernatant was collected. The process was repeated thrice with the residue. The supernatant was then mixed together and made upto 100ml using 80% acetone. The absorbance was read at 645nm and 663 nm using a visible range spectrophotometer. The chlorophyll content was calculated using the formula given below and is expressed in μg mg⁻¹ fresh weight.

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

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\[
\text{Chlorophyll b} = \frac{22.9 \times (A_{645}) - 4.68 \times (A_{663}) \times V}{1000 \times W}
\]

\[
\text{Total Chlorophyll} = \frac{20.2 \times (A_{663}) + 8.02 \times (A_{645}) \times V}{1000 \times W}
\]

A – Absorbance at specific wavelength, V – Volume of the extract, W – fresh weight of the tissue

3.8. Tissue Nutrient Analysis

3.8.1. Preparation of plant samples

Tillers collected from the representative plots by random sampling method were used for the nutrient analysis. The samples were then cleaned by washing in a mild detergent solution to remove the accumulated dust and other particles and followed by the distilled water wash. After washing, the samples were wrapped in a paper cover and kept for drying in a hot air oven at 65°C. The samples were kept until the weight has become constant. These were then ground in a Wiley mill and stored in labeled containers and used for the analysis.

3.8.1.1. Tissue Digestion

Tissue digestion was conducted as per the wet digestion method of Jackson (1958) and Garty and Fuchs (1982). 5grams of finely powdered plant samples from each concentration was weighed out and kept in a digestion tube with 15ml of acid mixture. The acid mixture was prepared by using the combination of a triple acid mixture of nitric acid, perchloric acid and sulfuric acid in the ratio 9:3:1. The digestion was carried out in an acid bath containing concentrated sulfuric acid at a low temperature of 125°C.

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for about 3 hours to obtain a clear digest. The clear digest was made up to the desired volume of 100ml with distilled water. This extract was used for the estimation of P and K. A blank was also maintained without the plant samples but with the triple acid mixture.

3.8.2. Estimation of Total Nitrogen

Total nitrogen was estimated as per the method of Jackson (1958) and Byju (2001). 250mg of dried, ground samples from each concentration was taken in the digestion tube and added approximately the same weight of salt mixture and 10ml of concentrated $\text{H}_2\text{SO}_4$. These were then kept in the Kel Plus digester and the temperature was adjusted to $410^\circ\text{C}$ and kept for one and a half hour for complete digestion.

The digested sample was transferred to the Kel Plus distillation apparatus and rinsed with distilled water. To this, 10ml of 40% NaOH was added and allowed the steam to pass through the sample and the ammonia thus formed was collected in an Erlenmayer flask containing 4% boric acid reagent. The samples were distilled for 8 minutes. The boric acid solution was then titrated with standardized HCl.

$$\% \text{ Nitrogen} = \frac{T \times \text{Normality of acid} \times 1.4}{\text{Sample weight}}$$

($T= \text{ml of acid used for sample titration} - \text{ml of acid used for blank titration}$)
3.8.3. Estimation of Phosphorus

Phosphorus content was estimated as per the method of Kitson and Mellon (vanadate-molybdate method) (Upadhyay and Sharma, 2002). From the triple acid digest, measured out 5ml and added 5ml of vanadomolybdate reagent and made up to 25ml using distilled water. The solution was kept over night at room temperature for incubation and then the yellow colour was read at 470nm on a spectrophotometer. A regression equation was developed using standard ppm solutions of potassium dihydrogen orthophosphate. The amount of phosphorous in the samples was calculated as follows.

\[ x = 299.86y + 2.533 \] (y is the absorbance of the sample)

\[ \% \text{ Phosphorous} = \frac{\text{value in ppm} \times \text{volume made up} \times \text{dilution}}{\text{Weight of the sample} \times 10000} \]

3.8.4. Estimation of Potassium

Potassium content was estimated as per the method of Byju, (2001). The triple acid digested extract itself was used for the determination of potassium following the flame photometer method. The made up extract is directly read in the flame photometer using appropriate filter. A standard solution of 10ppm was prepared with potassium chloride and was used as the standard. The calculation was done as follows.

\[ \% \text{ Potassium} = \frac{R \times \text{volume made up} \times \text{dilution factor}}{\text{Weight of sample} \times 10 \times 10000} \]

\[ (R – \text{Concentration of potassium in ppm}) \]

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3.9. Statistical Analysis

Statistical analysis was performed to test the significance at 5% and 1% levels using Analysis Of Variance (ANOVA) in Microsoft Excel. DMRT was also performed using MSTAT-C to evaluate the rank order and the results were represented with the superscripted alphabets.