Chapter-II
MATERIALS AND METHODS

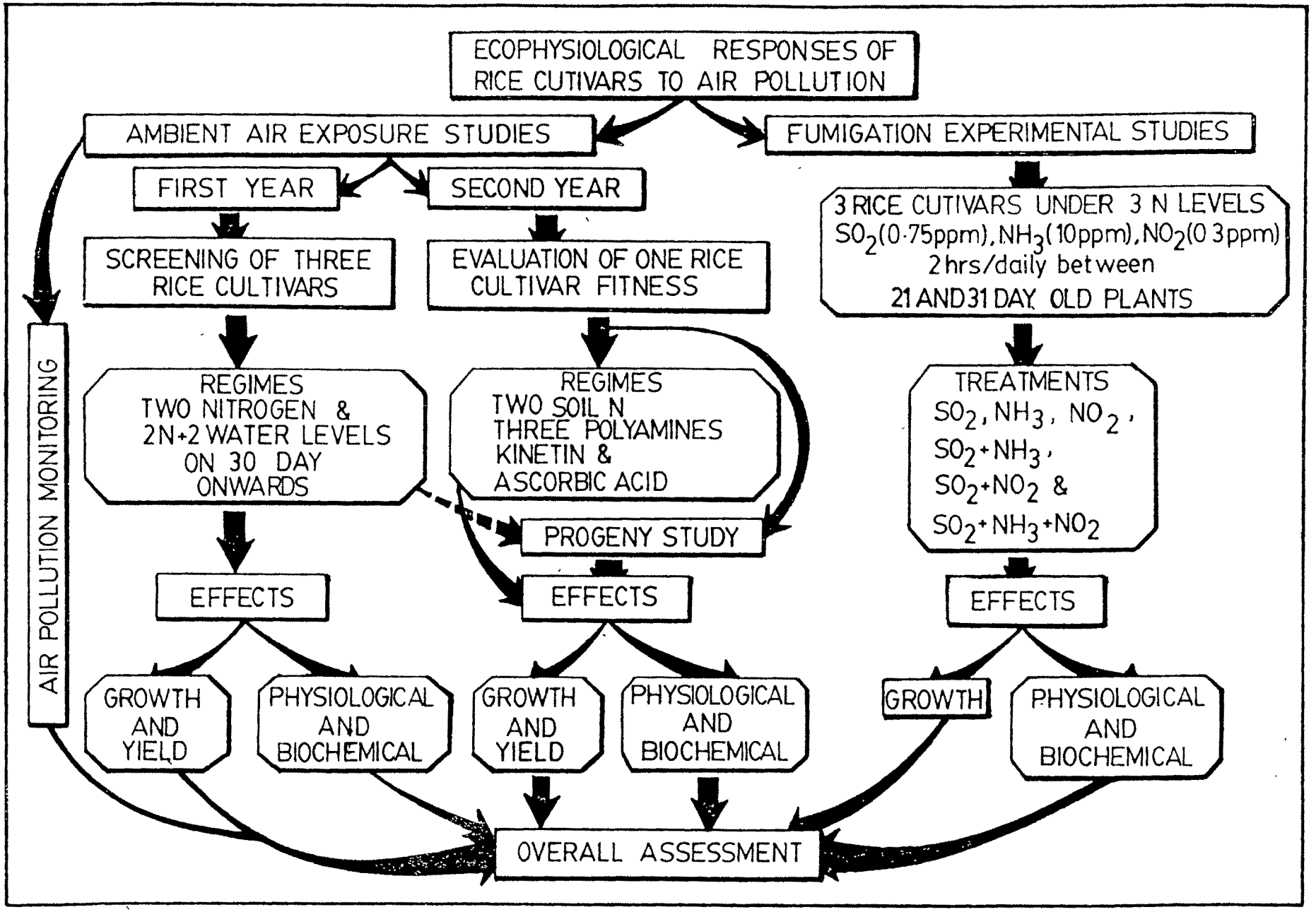


Fig. 2 Scheme to illustrate the overall work done.

MATERIALS AND METHODS

This chapter includes three sections namely

1. Experimental sites and air pollution monitoring,
2. Ambient air exposure studies and
3. Fumigation experimental studies.

2.1.0 EXPERIMENTAL SITES AND AIR POLLUTION MONITORING

2.1.1 Test Site

From the available air quality data, assessed by plotting contours using DEC 1030 system, it was clear that the north-west region of Baroda had maximum input on air quality compared to other parts of the VUDA area during 1982-1985 coinciding more or less the earlier assessment (Fig. 1; Patel **et al.**, 1986). For the present study a site near the Gujarat State Fertilizers Company Ltd. (GSFC) has been chosen as the test site because of the varieties of pollutants it discharges into the atmosphere amidst the rich agricultural lands (Fig. 3). The outside views of GSFC taken from the test site are shown in Plates 1 and 2.

2.1.2 GSFC and its Air Pollutants

The GSFC is located on the National Highway No. 8 (on the Baroda - Ahmedabad road) about 10 km north-western side away from Baroda. GSFC, established in 1965, manufactures Ammonia, Urea, Ammonium sulphate, Diammonium phosphate, Sulphuric acid, Phosphoric acid, Oleum and now Caprolactam. It also establishes itself to manufacture nylon chops, melamine and argon gas.

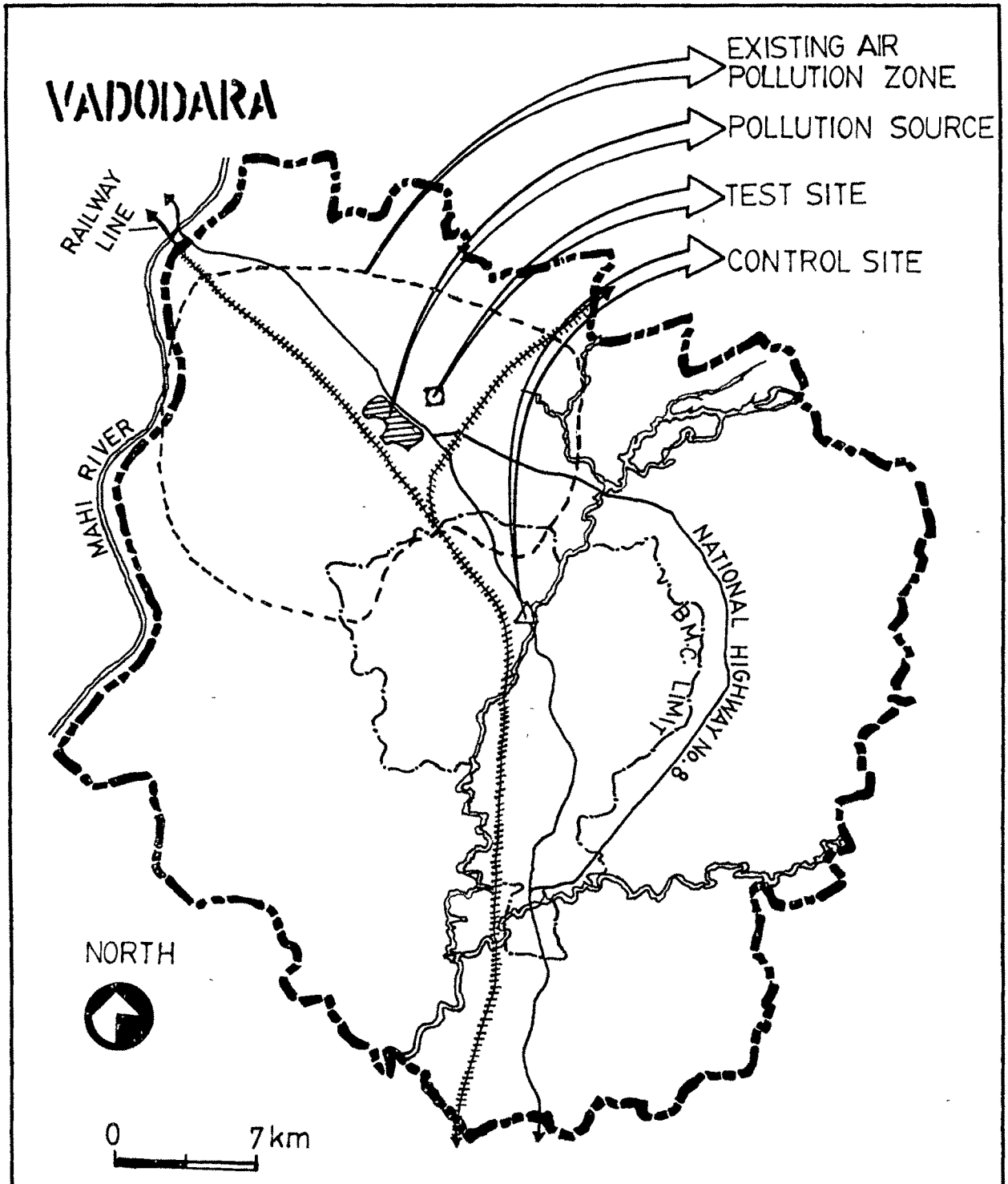


FIG: 3 MAP OF BARODA CITY SHOWING THE EXPERIMENTAL SITES

Table : 3 Air emission data of Gujarat State Fertilizers Co. Ltd., Baroda, India (Pachaiyapan and Nand, 1987)

Sr. No.		Stack Emission				Height calculation as per MINAS (m)
		Dia - meter	Height	Concentration. Mg/Nm^3	Quantity kg/d (Design)	
01.	Ammonia - I Flue gas	N.A	N.A	N.A	180	30
02.	Ammonia - II Flue gas	1.5	36.5	N.A	400	30
03.	Ammonia - III Flue gas	N.A	5.93 (above roof)	N.A	130	30
04.	Urea - I Prilling tower	N.A	48	92 (Urea dust) 62 (Ammonia)	260	30
05.	Urea - II Prilling tower	N.A	71	295 (Urea dust) 35 (Ammonia)	2560	40
06.	Phosphoric acid Plant Ball Mill	0.81	25	N.A	165	30
07.	DAP Plant Flume scrubber	1.37	27	N.A	138	30
08.	Old Sulphuric acid	1.30	53	400(SO_2) 200(SO_3)	570 285	41
09.	Oleum Stack	0.73	51.5	974(SO_2) 14(SO_3)	187 1.7	26
10.	New Sulphuric acid	1.22	52 (Above G.L.)	300(SO_2)	480	35
11.	Utility flue gas	2.4	25	165(NO_3)		

N.A indicates data are not available.

Plate-1 A view exactly from the study site showing SO_2 air emissions from the fertilizer plant.

Plate-2 Another view of the fertilizer plant showing NH_3 air emissions from the fertilizer plant.

The main raw materials are, associated gas, Naptha and Benzene, Sulphur, Rock Phosphate, Lubricants and fuel oils (Patel and Chokshi, 1978). A summary of the major air pollutants being discharged from GSFC are given in Table 3. Sulphur dioxide is the chief pollutant from this industry. There are two large, 50 m, height (raised to 100 m in 1987) chimneys that bring out the SO_2 . The concentration of gases, especially SO_2 is often very high and the visibility becomes very poor on the National highway passing near this industry, making it essential for the automobiles to move often with their lights on during the morning hours. At the extreme western side of the industry gaseous fluoride escapes in the process of manufacturing phosphate fertilizer. Manufacture of ammonia gas increase its ambient air concentration, at times causing irritation to eyes and throats.

2.1.3 Meteorological Observations

The mean values of maximum and mimimum temperature together with relative humidity and rain fall during the experimental period are given in Fig.4. The most important meteorological factor considered in the present investigation is the wind direction. The direction of wind was used as a main criteria for establishing experimental site. It is clear from Table 4 that the wind blows from south - west direction to a maximum number of days during monsoon season. So, the pollutants of the industry are carried to northern and north-eastern directions.

2.1.4.a Air Pollution Monitoring

Ambient air sampling may be considered as the collection of air samples

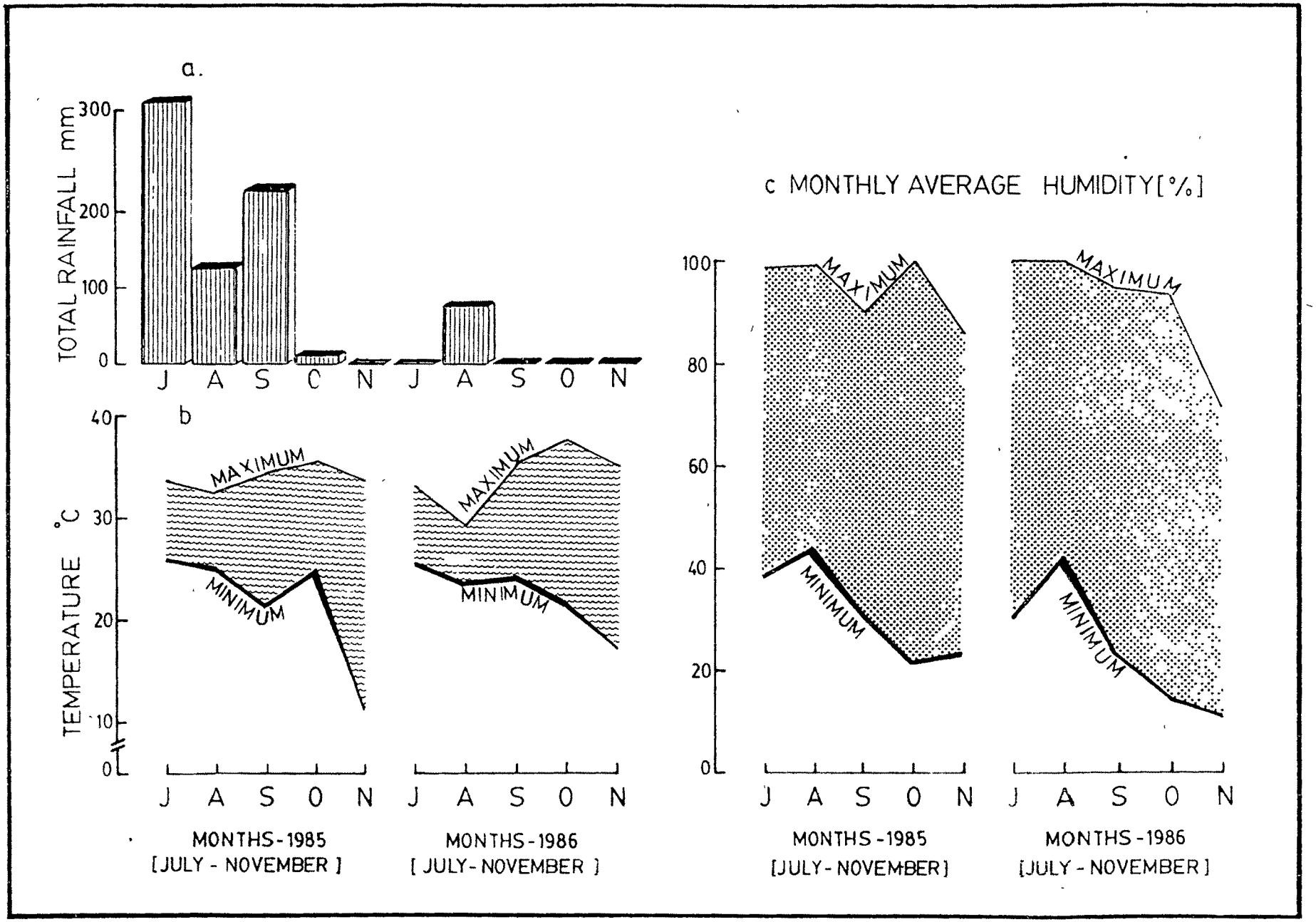


Fig. 4 Meteorological observations during field studies.

Table : 4 Average number of days, speed and wind direction during growth period of 1985 and 1986.

	1985					1986				
	July	Aug.	Sept.	Oct.	Nov.	July	Aug.	Sep.	Oct.	Nov.
North-east	0.0	0.0	0.0	4.2	3.2	0.0	0.0	0.0	0.0	4.0
East	0.0	0.0	0.0	0.2	0.4	0.0	0.0	0.0	0.8	0.6
South-east	1.0	0.2	0.0	0.4	0.2	0.0	0.8	0.4	1.0	0.2
South	5.4	3.4	2.4	1.0	0.2	1.0	1.6	2.0	1.4	0.2
South-west	22.0	24.2	17.6	3.0	1.4	25.6	20.2	14.8	7.0	1.4
West	0.4	1.8	5.6	0.0	0.4	1.2	1.2	2.0	0.8	0.6
North-west	0.0	0.0	0.0	4.4	2.4	0.0	0.0	0.6	0.0	6.4
North	0.4	0.0	0.0	1.8	1.2	0.0	0.0	0.2	1.0	2.4
Calm	0.0	0.4	1.0	14.0	19.0	0.0	2.4	3.6	12.2	12.2
Miscellaneous	1.0	1.0	3.4	2.0	1.6	3.2	3.8	6.5	5.8	2.0
Average speed km/hour	7.1	6.9	5.9	5.7	0.9	9.5	5.4	5.0	3.3	1.6

in any unconfined location exposed to the atmosphere. Air monitoring survey near the test-site between March 1982 and 1985 showed that the ambient air pollutants were found to be chronic. The annual mean of SO_2 ranges between 11 and 19.3 $\mu g/m^3$ with a maximum daily average of 143.9 $\mu g/m^3$. For NO_x it was between 37 and 79.3 $\mu g/m^3$ with maximum of 210 $\mu g/m^3$, daily average. Suspended particulate matter was found to be between 212 and 534 $\mu g/m^3$ with a maximum daily average of 870 $\mu g/m^3$ (Patel **et al.**, 1987).

In the present study, for air monitoring, the well established and sufficiently precise and accurate methods for Sulphation rate (Wilson and Mc Connel, 1934), Nitrogen dioxide (Saltzman, 1954), Ammonia (Weatherburn, 1967) and Fluoride (Hendrickson, 1968) were adopted for the analysis.

The concentrations of pollutants during the study period are given in Table 5. In the first year (1985) study the levels of pollutants were high at the test site in the month of July except fluorine, when compared to the other months of study. The average sulphation rate for the growth period was between 0.78 and 1.07 mg $SO_4^-/cm^2/day^{-1}$. The NO_2 was between 19 and 38 $\mu g/m^3$, NH_3 was between 1,54 and 1.76 mg $/m^3$ and fluorine was between 0.33 and 0.52 $\mu g/cm^2/month^{-1}$. In the second year (1986) the sulphation rate was increased at the test site. The average sulphation rate was between 0.88 and 1.25 mg $SO_4^-/cm^2/day^{-1}$, NO_2 was between 15 and 44 $\mu g/m^3$, NH_3 was 1.42-1.84 mg/m^3 and F was between 0.27 and 36 $\mu g/cm^2/month^{-1}$. The methods adopted are discussed hereunder.

Table : 5 The levels of air pollutants during the study period at the control and test sites as expressed in the monthly average values.

		1985						1986				
		July	Aug.	Sept.	Oct.	Nov.	Average	July	Aug.	Sep.	Oct.	Average
Sulphation rate mg $SO_4/cm^2/day^{-1}$	C	0.12	0.24	0.26	0.24	0.24	0.22	0.10	0.19	0.17	0.23	0.173
	P	1.07	0.86	0.98	0.78	1.06	0.95	0.88	1.13	1.25	1.17	1.11
NO_2	C				Negligible					Negligible		
$\mu g/m^3$	P	38	24	21	19	24	25.2	29	15	19	44	26.8
NH_3	C				Negligible					Negligible		
mg/m^3	P	1.76	1.63	1.66	1.54	1.63	1.64	1.42	1.69	1.84	1.76	1.68
F	C				Negligible					Negligible		
$\mu g\ F/cm^2/month$	P	0.35	0.33	0.41	0.52	0.39	0.40	0.27	0.31	0.31	0.29	0.308

2.1.4.b Sulphation Rate

The concentration of sulphation rate gives a cumulative index of the sulphur containing compounds in the atmosphere. Inspite of the newer techniques available for air monitoring this method has been used by various authors in evaluating long term effects of SO_2 on plant growth and productivity (Thomas and Davidson, 1961; Huey, 1968; Cr. Godzik and Krupa, 1982). In India, this method with slight modification was used by Zutshi and his co-workers (1975) at Bhabha Atomic Research Centre, Bombay, for monitoring ambient air.

Preparation of Lead Peroxide Candle

A gum-tragacanth mucilage was prepared by dispersing 2.0 g of gum-tragacanth in 10 ml of absolute alcohol. It was constantly stirred and 190 ml of distilled water was added, 8 g of lead peroxide was homogenised in suitable quantity of this mucilage, 100 cm^2 reactive surface area was prepared by wrapping cotton gauge around a culture tube where the lead peroxide mixture was uniformly applied with the help of spatula. The prepared candles (cylinders) were kept in a dessicator for drying. Later, candles were taken to the test site, where they were kept inside the louvered box. The box containing the candle was placed approximately at a height of 6 m. A candle at control site and one candle in a separate dessicator were kept for references.

Analysis of Lead Peroxide Candle

After exposure for a month the candle were brought back to the laboratory by replacing new ones. The exposed lead peroxide from the candle was removed and placed in a beaker with 5 g of anhydrous sodium carbonate in 100 ml of distilled water. The treated content was allowed to stand, with

occasional sirring at least for 3 hours and was boiled for half an hour, keeping the volume nearly constant by addition of distilled water. After filtering the mixture through Whatman No. 42 filter paper, the filtrate was acidified with conc HCl and sulfate was precipitated with 10% barium chloride solution. The mixture containing $BaSO_4$ was filtered on ashless filter paper. The filter paper along with its content was transferred to a crucible and ignited. It was cooled and weighed. The additional weight was the amount of $BaSO_4$. In a similar way the amount of $BaSo_4$ determined on the control cylinder or candle was determined. Before calculating the amount of SO_2, the weight of the exposed candle was substracted by the control cylinder. The results are reported as sulphation rate in mg SO_4/100 cm^2 of exposed surface per day.

2.1.4.c Air Sampling

Sampling of air for the analysis of NO_2 and ammonia was done as described under hand-operated vacuum pumps by Hendrickson (1967). Gas tight syringes (Hemilton-USA) were used for this purpose. In the vessels containing absorbing solutions for NO_2 and NH_3 the ambient air was injected for 30 minutes in each, daily.

2.1.4.d. NO_2 - Quantification

2 ml of butyl alcohol was added per litre of 0.1 N sodium hydroxide solution to increase foaming and to assist in trapping the nitrogen dioxide. The samples of absorbing reagents were transferred into 50 ml of Nessler's tubes.

One drop of 1% hydrogen perodixe solution was added and mixed to oxidize the dissolved SO_2 to sulphate, thereby preventing SO_2 interference. 10 ml of diazotising reagent (4 g of sulfanilamide dissolved in 250 ml of distilled water containing 5 ml of phosphoric acid/and 1 ml of (N) - (N-Naphthyl) ethylene-diamine dihydrochloride reagent (1mg/1 ml) was added and diluted to 50 ml. The solution was mixed well and kept for 30 minutes. The optical densities were determined in a CARLZEISS Spekol colorimeter at 550 nm, using reagent blank as the reference. Observations noted were direct observations from single sampling vessels without applying the absorption efficiency factor. Concentration of samples were read from plotted standard curve (using sodium nitrite) as ppm and after calculations the concentraction NO_2 was expreseed as $\mu g/m^3$.

2.1.4.e Ammonia - Quantification

0.02 N of sulphuric acid was used as the absorbing medium for ammonia. Two reagents namely A and B were used. Reagent A constituted Phenol plus nitro-prusside : 5 g of phenol with 25 mg of sodium nitroprusside/500 ml of solution. Reagent B constituted alkaline hypochlorite : 2.5 g of sodium hydroxide, 4.2 ml of sodium hypochlorite to 500 ml of solution. All solutions were prepared from distilled water free of ammonia.

A suitable aliquot of NH_3 absorbed medium was added with a 5 ml of reagent A into a test tube. The test tube was shaken vigorously to mix. 5 ml of reagent B was added followed by thorough mixing. It was allowed for 20 minutes for colour development at 37 °C. The absorbance was measured in room temperature at 625 nm using spekol colorimeter with a reagent blank for reference. Ammonium sulphate was used for standard and the values are expressed in mg/m^3.

2.1.4.f. Fluoride

For the determination of fluoride concentrations into ambient air, a simple technique had been used as quoted by Hendrickson (1968). An approach which is satisfactory for many purposes is to obtain cumulative indications over a period of time by simplified techniques (Hendrickson, 1968). No quantitative measure of the concentration of polluting material per unit volume of air may be obtained by such methods, but satisfactory information for many purposes may be secured. The unit cost of such techniques is practically insignificant when compared with more elaborate procedures and equipment.

Whatman No.1 filter paper (12.5 cm diameter) were soaked in lime suspension consisting of 28 g of low fluoride lime per litre of water. The papers were dried and exposed in a group of six in shelters which permitted adequate air movement but protected the papers from the weather. Six treated papers were mounted on a rack in vertical position, spaced 25 mm apart. A month after exposure the papers were ashed (The analysis was done by following SCOPE 6 (1975) method). The results were expressed in $\mu g/F/cm^2$/ month.

The reagents used were alizarine complexone (o.385 g of reagent dissolved in a minimum amount of dilute NaOH and diluted to 1 litre of acetate buffer, pH 4.3, containing 34 ml of glacial HOAC and 18.8 g of anhydrous NaOAC/litre) and lanthanum nitrate buffer (0.866 g of La $(NO_3)_3$ was dissolved with 10.2 g of anhydrous NaOAC in 400 ml of distilled water, 222 ml of glacial HOAC and 890 m eq of HCI were added and diluted to 1 litre with pH 4.1 to 4.2 . With an aliquot of plant samples 10 ml of alizarine complexone, 10 ml of lanthanum buffer and 25 ml of acetone were added and diluted to 100 ml with H_2O and

O.D. was read at 618 nm using spekol colorimeter. F^- concentration was established from an appropriate working curve.

2.2.0 AMBIENT AIR EXPOSURE STUDIES

This section inturn consists of two years seasonal studies : 2.2.1 I year studies and 2.2.2. II year studies. Both year studies include 1. Experimental design, materials and regimes, 2. Growth and yield studies, and 3. Physiological and Biochemical studies.

2.2.1 I YEAR STUDY

2.2.1.1 a Design, Materials and Regimes

The present study was carried out by potculture method to establish the differences between the treatments under comparison and to estimate quantitatively main effects of tested factors and factor interactions on the growth and crop yield. The experimental plant is a mandatory feature of the potculture trial. The pots were kept in a 2 x 3 x 2 x 2 (2 environments; 3 cultivars; 2 nitrogen and 2 nitrogen + water regimes) factorial design as described by Dospekhov (1984).

2.2.1.1 b Materials

The seeds of rice (**Oryza sativa** Linn.) varieties Coimbatore 43 (CO 43) and Tirurkuppam 9 (TKM 9) from the Tamil Nadu Agricultural University,

Coimbatore and Gujarat Rice 3 (GR 3) from the Main Rice Research Station. Navagam, India, were procured for this experiment.

2.2.1.1c Germination

Seeds of CO 43, GR 3, and TKM 9 rice cultivars were washed in distilled water, surface sterilized with 0.1% mercuric chloride for 3 minutes, washed repeatedly to be free of $MgCl_2$ and kept in an incubator at 28 ± 1° C temperature in moistened filter papers. Three day old pre-germinated seeds were sown in the puddled soils of polythene containers containing 8 kg of garden soil. The mineral composition of the saturation extract of the soil used was as follows, analysed by standard procedures (Chopra and Kanwar, 1980; Jackson, 1967): pH 6.3, electrical conducitivity 0.6 m mhos/cm^3; sodium 1.6, potasium 1.08, calcium 4.0 magnesium 2.7, chloride 0.55, bicarbonate 21.0, and sulphate 1.16 m eq/1 and nitrogen 0.25 mg/g dry soil.

The experiment was carried out during Kharif season (July-November) of 1985, by placing the pots approximately 500 m away from the SO_2 point sources of GSFC in the downwind direction. A view from the study site showing the point source is shown in plate 3. Controls were maintained, in á rellatively least polluted area, Botany department net-house with uniform edaphic factors and cultural practices. Practically the candidate himself had carried water to the test site from the department, daily.

2.2.1.1d Crop Tending

Tending of crop in the test site and control site were done carefully

Plate-3 A focus on the point source (Chimney) of the fertilizer plant from the test site.

and uniformly in due time. Thinning of plants was done at 15 and 30 days after seeding thereby allowing 8 plants and 5 plants for further study, respectively.

2.2.1.1e Imposition of Regimes

30 day-old plants were imposed to regimes under two heads : (i) Normal Watered (WN) and (ii) Water Stressed (WS), The latter was accomplished by maintaining half field capacity till the harvest by weight method. To avoid changes during rainy days transparent polythene sheet tents were used above the plants as a precautionary measure. Both WN and WS inturn constitute two regimes as under :

a. Normal Nitrogen (NN) : Uniform dressing of nitrogen was by urea (46% N) containing 96 mg of N/kg/dry soil, in three equal amounts at 30,55 and 70 day-old plants.

b. High Nitrogen (HN) : Urea containing 224 mg of N/kg dry soil, in three equal amount at 30, 55 and 70 day old-plants.

2.2.1.2 Growth and Yield Studies

2.2.1.2a Early Growth Studies

The height of the plants was measured above the ground level at different stages of growth after 15 days and onwards. Total photosynthetic leaf area was measured initially using planimeter and later by conversion factor and dry matter accumulation was recorded after keeping the shoot systems at 80° C to a constant weight.

2.2.1.2b Later Growth Studies

(i) Growth

Morphological parameters such as height of the plants, number of tillers per plant, number of leaves per plant, and total photosynthetic leaf area were determined from the shoot portions of 55, 70 and 85 day old plants. The sampling was done randomly as per Dospekhove (1984). Fresh weight and dry weight of shoot portions were also determined from the samples.

(ii) Yield and Yield attributes

Dates of panicle initiation were recorded by numbering the plants with tags. At maturity, yield components were measured as described by Yoshida **et al.**, (1976). The yield components, panicle length, number of panicles per plant, number of spikelets per panicle, number of filled and unfilled grains per plant, sterility index of grains, dry weight of filled (economic yield) and unfilled grains per plant, straw weight per plant, biological yield, harvest index and the weight of 100 grains were analysed. The biological yield (total dry standing crop) and the harvest index (grain yield/biological yield) were calculated as described by Donald and Hamblin (1976). All measurements were taken from five replicates, each consisting of two plants. Data were statistically analysed using analyses of variance (ANOVA) with the guidance of Statistics Department of M.S. University of Baroda.

2.2.1.3 Physiological and Biochemical Studies

This includes the determination of chlorophylls, ascorbic acid, nitrogenous fractions (free amino acids, free proline, protein, nucleic acids, total quaternary ammonium compounds and total nitrogen), activities of different enzymes, glutathione,

total sulphur, fluoride, total phenolics, total soluble sugars and starch from shoot portions, as described hereafter. All assays had three replications and standard error was worked out. Enzymes and glutathione assays had only two replications.

2.2.1.3a Chlorophylls

The contents of chlorophyll were extracted from the third leaf from the top of rice plant. In rice plants third leaves from the top reported to be sensitive to SO_2 pollution when comparing other leaves (Valenzona, et al. 1978). The extraction was with ice cold 80% (v/v) acetone and the determination was from the optical densities measured at 663 nm and 645 nm in a spekol colorimeter and chlorophyll 'a,' chlorophyll 'b' and total chlorophyll were calculated as per Arnon (1949).

2.2.1.3b **Ascorbic Acid**

Determination of ascorbic acid was by following the method of Jayaraman (1981)., 500 mg of fresh leaf material was ground with 15 ml of 5% metaphosphoric - 10% acetic acid solution. The homogenate was filtered. To a 12 ml aliquot of the filtrate 0.75 g of activated charcoal was added and vigorously shaken. The sample was filtered in Whatman No.1. To a 4 ml aliquot of the filtrate in a test tube 1 drop of 10% thiourea solution and 1 ml of 2,4 - dinitrophenyl hydrazine reagent (2g of reagent in 100 ml of 9N H_2SO_4 and filtered) was added. The tubes were placed at 37° C water bath for exactly 3 hours. The tubes were cooled in ice. 5 ml of 85% H_2SO_4 was added dropwise with constant stirring. After 30 minutes the colour development was measured at

540 nm using Carlzeiss colorimeter. The ascorbic acid content was calculated from a plotted standard curve.

2.2.1.3c Free Amino Acids

The method employed for the extraction of free amino acids was essentially that of Singh **et al.**, (1973). 200 mgs of leaf tissues were homogenized with 2 ml of methanol-chloroform-water (MCW) (12:5:1) at room temperature. The homogenate was centrifuged to collect a clear supernatant. The residue was shaken with a further 2 ml of MCW for 5 minutes and centrifuged. The supernatants were combined, and 1.5 ml of water with 1 ml of chloroform was added to break the stable emulsion formed during extraction. The lower phase containing chlorophyll was separated. The upper phase was diluted with 10 ml water and used for estimation of free amino acids.

The method used for the estimation of free amino acids was essentially that of Rosen (1957), ninhydrin method, using glycine for standard.

2.2.1.3d Free Proline

500 mg of fresh leaf materials were homogenized in 5 ml of 3% aqueous sulfosalicylic acid and two ml aliquot of the filtered homogenate was allowed to react with 2 ml of acid ninhydrin (3.75 g ninhydrin + 900 ml glacial acetic acid + 60 ml of 6 M H_3PO_4 warmed until dissolved and stored at 4°C) and 2 ml of glacial acetic acid for 1 hour at 100°C. The chromophore extracted with 5 ml of toluene was estimated for proline content using a Carlzeiss colorimeter following the method of Bates **et al.**, (1973). Calculations were made using a standard curve.

2.2.1.3 eProtein

The protein was extracted as described by Prisco and Vieira (1976). 200 mgs of fresh leaf materials were finely ground in 0.01 M Na-K phosphate buffer, pH 7.6, containing 0.1 M NaCl. The ratio of tissues to the grinding medium was 1:10 (w/v). The homogenate was centrifuged at 3000 r.p.m. for 10 minutes and the supernatant was assayed for total protein. An equal volume of 10% TCA was added to this supernatant. After centrifugation the pellet was resuspended in a volume of 0.1 N NaOH equal to the initial supernatant and the insoluble protein was assayed. Soluble protein was determined as the difference between total and insoluble protein. The determination of protein was by the modified Lowry **et al.**, method as described by Hartree(1972).

2.2.1.3 f Nucleic acids

Extraction and estimation of RNA and DNA were by following the method of Jayaraman (1981), 200 mgs of dry fine-powder of shoot system was suspended in 10 ml of cold 10% TCA for 30 minutes and centrifuged at 3000 r.p.m. for 10 minutes. The supernatant was discarded. The pellet was again washed with 5 ml of cold 10% TCA and recentrifuged. The supernatant was discarded. The precipitate was resuspended in 5 ml of ethanol-ether mixture (1:1) and centrifuged and again the supernatant was discarded. The precipitate was dissolved with 5 ml of 0.5 NaOH and kept at 37°C for 18 hours. The sample was once again centrifuged. The supernatant was precipitated with equal volume of 10% TCA and recentrifuged. The precipitate was discarded and the supernatant was taken for the estimation of ribose sugars.

The precipitate obtained from centrifugation after an addition of 0.5 N NaOH, was suspended with 1 ml of perchloric acid. The sample was heated in a boiling water bath for 1 hour, cooled and centrifuged. The precipitate was discarded and supernatant was taken for the estimation of deoxyribose sugars. The ribose and deoxyribose sugars were assayed using orcinol and diphenylamine reagent, respectively.

2.2.1.3 g Total Quaternary Ammonium Compounds, QACs

500 mg of finely ground dry plant shoot portion was mechanically shaken with 20 ml of deionized water for 24 hours at 25°C. Time required for this step was determined by extracting the plant samples for 1,4,16,24 and 48 hours. The samples were then filtered and the filtrates were stored in the freezer until analysis (Grieve and Grattan, 1983).

Thawed extracts were diluted1:1 with 2N H_2SO_4. The acid potassium triiodide solution for total QACs was prepared by dissolving 7.5 g resublimed iodine and 10 g potassium iodide in 1 M HCl and filtering (Speed and Richardson, 1968). Precisely 0.2 ml of acid potassium triiodide reagent was added to an aliquot of sample containing between 10 and 150 µg of QACs in water. The mixture was shaken and left for atleast 90 minutes in an ice bath with intermittent shaking 2 ml ice-cold water was added rapidly to the mixture to reduce the absorbance of blank and improve replication. This was quickly followed by 10 ml 1,2-dichloroethane in ice, and the 2 layers mixed well and kept at low temperature at 4°C (Storey and Wyn Jones, 1977). The absorbance of the lower organic layer was measured at 365 nm in a Shimadzu Spectrophotometer. The results are expressed as glycinebetaine equivalent by using glycinebetaine for standard value.

2.2.1.3 h Total Nitrogen

The total nitrogen was estimated as described by Umbriet **et al.,** (1959). 50 mgs of dry shoot powder was digested with 2 ml of 2N H_2SO_4 containing copper selenate (20 mg/100 ml of 2N H_2SO_4) and the final volume made up to 25 ml. An aliquot of the sample was taken and the nitrogen content was estimated using double iodide reagent, colorimetrically, using Systronics colorimeter.

2.2.1.3 i Studies on the Activity of Enzymes

The estimation of maximum catalytic activities of enzymes in plant tissues can contribute significantly to the elucidation of metabolic pathways and the regulatory mechanisms which control them. Three weeks after the imposition of regimes shoot systems were harvested for the enzymatic studies. The fully expanded third leaves from the top were taken for all the enzymatic extractions. All the assays had two replications.

(i) Peroxidase (EC 1.11.1.7)

500 mg of leaf materials were homogenised with 3 ml of phosphate buffer (0.1 M, pH 6.0) in a chilled mortar and pestle. The extract was squeezed through two layers of cheese cloth and centrifuged at 10,000 r.p.m. for 15 minutes. The supernatant served as the enzyme source for peroxidase and polyphenol oxidase. All operations were carried out at 0-4°C.

Peroxidase activity was assayed according to the procedure given in "Enzymes" (Published by Worthington Biochemical Corporation, Freehold, New

Jersy, 1963). The assay system contained 6 ml of substrate (1 ml of the stock containing 30 per cent H_2O_2, in 100 ml, was diluted to 100 ml with phosphate buffer, pH 6.0), 0.1 ml enzyme, 0.05 ml 5% O-dianisidine in methyl alcohol and distilled water to make the final volume to 7 ml. The reaction was initiated by the addition of enzyme and the colour development was read in spekol colorimeter at 460 nm. The absorbancy increase was noted at 15 seconds intervals. The activity was expressed as units per minute per mg protein. A unit of the enzyme activity was the increase in 0.1 O.D. at 460 nm.

(ii) Polyphenol Oxidase (EC 1.10.3.1)

The enzyme source was the same as used for peroxidase assay. The method for polyphenol oxidase assay was essentially that of Taneja and Sachar (1974). The assay system contained 4 ml catechol (10 mg/ml) as substrate, 0.2 ml enzyme and phosphate buffer (0.05 M, pH 6.7) to make up the volume to 6 ml. Omission of enzyme from the incubation mixture served as blank. Prior to mixing, all the ingredients were maintained at 37°C. The reaction was initiated by the addition of enzyme and the colour development was measured at 430 nm in a CARLZEISS colorimeter. The activity was expressed as units increased per minute per mg protein. A unit of the enzyme activity was the increase in 0.1 O.D. at 430 nm.

(iii) Amylase (EC 3.2.1)

One g of leaf materials were homogenised at 0-2°C with a pinch of acid washed glass powder and 5 ml of 0.01 M acetate buffer (pH 4.6). The

homogenate was centrifuged at 6,000 r.p.m. for 15 minutes at 0 to 2°C. The supernatant was used as the source of enzyme.

The assay system contained 0.5 ml of 0.01 M of acetate buffer (pH 4.6), 1 ml of 1% soluble starch in acetate buffer (0.01 M pH 4.6), 0.2 ml enzyme and distilled water to make up the final volume to 5 ml (Paleg, 1960). The reaction was allowed to proceed at 30°C for 10 minutes. An aliquot of 1 ml of the reaction mixture was withdrawn at the end of 10 minutes and added to 1 ml of 3,5-dinitrosalicylic acid reagent (Bernfeld, 1951) to terminate the reaction. This was incubated for 10 minutes in boiling water-bath, cooled and diluted with 10 ml of water. Colour developed was read at 540 nm in a CARLZEISS colorimeter and compared with a standard curve of maltose. The enzyme activity was expressed as µmoles of maltose released per 10 minutes per mg protein.

(iv) Invertase (EC 3.2.1.26)

The crude extract of amylase was used for invertase assay. The method of enzyme assay was that of Malik and Singh (1980). The assay system contained 0.4 ml of 0.1 M acetate buffer (pH 4.6), 0.25 ml of 0.4 M sucrose and 0.35 ml of enzyme extract to give a total volume of one ml. In control tubes sucrose solution was added only when enzyme was inactivated by boiling for about 5 minutes. After, incubation at 30°C for 1 hour, 1 ml of 3,5-dinitrosalicylic acid reagent was added. The tubes were placed in boiling water bath for 10 minutes. Then the contents were diluted to 10 ml and read at 560 nm in a CARLZEISS colorimeter. Glucose was used as a standard to calculate the released reducing sugar. The activity was expressed as µ moles of reducing sugar produced per hour per mg protein.

(v) Phosphorylase (EC 2.4.1.1)

One g of leaf materials were homogenised with little amount of glass powder in a chilled mortar and 10 ml of cold distilled water was added. The homogenate was centrifuged at 6,000 r.p.m. for 15 minutes at 0 to 2°C. The supernatant was used as the source of the enzyme.

Phosphorylase activity was assayed using the modified method of Ozbun **et al.,** (1973). The reaction mixture contained 0.2 ml of 5 % starch solution, 0.5 ml of 0.5 M citrate buffer (pH 6.5), 1.0 ml enzyme and water in a total volume of 2.5 ml. The enzyme - buffer mixture was equilibrated at 35°C for 5 minutes and 1.0 ml of 0.1 M glucose -1-phosphate was added. The mixture was then incubated at 35°C for 10 minutes before terminating the reaction by the addition of 5 ml of 5% TCA. The reaction mixture was centrifuged to separate the supernatant from the pellet and 1.0 ml of the aliquot was used in the determination of inorganic phosphate by the method of Fiske and Subbarow (1925). The activity was expressed as μ moles of phosphate released per 10 minutes per mg protein.

(vi) Acid Phosphatase (EC 3.1.3.2)

The method employed for the extraction and assay of acid phosphatase was essentially that of Hasson-Porath and Poljakoff-Mayber (1971). One g of leaf materials were homogenized with 7 ml of cold Tris-Maleate buffer (0.1 M, pH 7.0), containing 0.1 M sucrose and 0.003 M magnesium sulphate. The homogenate was centrifuged at 10,000 r.p.m. for 20 minutes in a refrigerated

centrifuge at 0-2°C. The supernatant was collected and the residue was washed once with 3 ml of the same buffer. The combined supernatant was used for the assay of acid phosphatase.

The assay system of phosphatase contained 2.5 ml of Tris-Maleate buffer (0.1 M, pH 5.0), 1 ml of p-nitrophenyl phosphate (8 μ moles/ml) and 0.5 ml of crude enzyme extract. The reaction was started by the addition of enzyme. The reaction mixture was incubated at 30°C for 30 minutes before terminating the reaction by the addition of 3 ml of 0.2 N NaOH. The samples were allowed to stand for 1 hour at room temperature and colour development was measured colorimetrically at 410 nm using CARLZEISS colorimeter. p-Nitrophenol released during the enzyme activity was calculated from a standard curve. The activity was expressed as μ moles of p-nitrophenol released per 30 minutes per mg protein.

(vii) Nitrate reductase (EC 1.6.6.1)

One g of leaf materials were extracted with a pinch of neutral glass powder in 6 ml of the extraction medium (1 mM EDTA, 10 mM cysteine, 25 mM potassium phosphate adjusted to a final pH of 8.5 with KOH) using chilled mortar and pestle at 0-3°C. The macerate was passed through a 4 layers of cheese cloth and the filtrate was centrifuged at 10,000 r.p.m. for 15 minutes at 0°C. The supernatant was used as the crude enzyme preparation.

The activity of nitrate reductase was assayed essentially following the procedure described by Hageman and Flesher (1960). 3 ml reaction mixture

contained 1 ml of 0.1 M potassium phosphate buffer, pH 7.5, 0.2 ml of 0.1 M KNO_3, 0.5 ml of 2 mM NADH, 0.2 ml of enzyme extract and distilled water. The reaction was carried out at 30°C for 60 minutes and terminated by the addition of 1% (w/v) sulphanalamide in 3N HCl and the nitrite formed was estimated according to Snell and Snell (1949). The activity of the enzyme was expressed as n moles of NO_2 - formed per hour per g fresh weight.

2.2.1.3. j Glutathione

One g of leaf material was homogenized in a chilled mortar and pestle with 3 ml of 1 % metaphosphoric acid. The homogenate was centrifuged in cold condition (0 to 4°C) at 5,000 r.p.m. for 15 minutes and the supernatant was decanted. With 2 ml of the above said aliquot 6 ml of saturated sodium chloride solution was added and kept at 20°C for 15 minutes. For reagent blank 2 ml of 2% metaphosphoric acid in saturated NaCl solution was used instead of sample. After the incubation period one ml of solution containing 1.5 M sodium carbonate and 0.067 M sodium cyanide was added to the reaction tube. After thorough mixing one ml of 0.067 M sodium nitroprusside solution was added. With the addition of nitroprusside the readings were taken immediately (within 10 seconds) at 540 nm in a CARLZEISS colorimeter. The readings were compared against a standard curve and expressed as µg of glutathione per g fresh weight as per Grunert and Phillips (1951).

2.2.1.3. k Total Sulphur

500 mg dry and fine powder of leaf material was used for wet digestion.

One ml of ammonium metavanadate (1.5mg/1 ml perchloric acid) solution, 0.5 ml potassium dichromate solution (30 mg/ml water) and 5 ml of acid mixture (perchloric acid and nitric acid, 4:6) were added and heated in a heating mantle till the material residue in the flask becomes red in colour (Garrido, 1964). The flask was removed and washed with little water and the solution was transferred to 100 ml volumetric flask. The solution was diluted to 100 ml and the aliquots were taken for sulphur estimation.

The procedure for estimation of sulphur was essentially followed as per Hunt (1980) using turbidimetry. To the 2 ml aliquots, 10% HCl was added for further dilution. To 5 ml of the acidified solution 3 ml of Barium chloride-Tween 80 solution (26.7 g of barium chloride and 133 ml of Tween 80 solutions dissolved in 800 ml of water and diluted to 1 litre was allowed to stand for a minimum of 24 hours before use) was added, mixed thoroughly and kept aside for 40 minutes. It was mixed again prior to reading the abosorbance of solution at 420 nm in a 1 cm cell against water as reference using Systronics colorimeter. The readings were calculated from a standard curve using potassium sulphate.

2.2.1.3. 1 Fluoride

The procedure involved for the extraction of plant materials was as described by Mc Quaker and Gurney (1977). 500 mg of dry and fine powder was taken in a nickel crucible and was moistened with little water. This was followed by the addition of 6 ml of NaOH (67 g/100 ml water) solution. The crucible was then tapped slightly so as to disperse the sample uniformly in

the sodium hydroxide solution. If this had been achieved, the samples were placed in an oven, set to 150°C, for 1 hour, and then removed. After the NaOH had been solidified, the crucibles were placed in a muffle furnace set to 300°C. The temperature was then raised to 600°C and the sample was fused at this temperature for 3 minutes. Later, the sample had been removed from the muffle furnace and allowed to cool, 10 ml of distilled water was added to the samples. The samples were then heated slightly so as to facilitate the dissolution of the sodium hydroxide cake. Next, about 8 ml of conc HCl was slowly added with stirring, so as to adjust the pH 8-9. This was transferred to a 100 ml volumetric flask and diluted to volume and filtered through dry Whatman No. 40 filter paper. The filtrate was used for F estimation. The estimation of F was by following the method of SCOPE 6 (1975) as described earlier (2.1.4. f).

2.2.1.3. m Total Extractable Phenolic Compounds

Phenolic compounds were extracted by grinding 50 mg of dry plant materials using a chilled mortar and pestle with an aliquot of chilled 80% (v/v) ethanol. The homogenate was centrifuged at 8,000 r.p.m. for 15 minutes. The supernatant was collected and residual pellet was extracted twice again. The supernatant of all three extractions was pooled and volume was made upto 25 ml with 80% ethanol.

The ethanol extractable phenolic compounds were estimated by Folin method of Swain and Hillis (1959). A suitable aliquot of the ethanolic extract was diluted with distilled water to 8.5 ml and after adding 0.5 ml of Folin-phenol reagent, the contents were mixed well. Three minutes later 1 ml of saturated sodium carbonate solution (1 g/ 3 ml water) was added and the mixture

was shaken thoroughly. The colour was allowed to develop for 60 minutes and then read at 725 nm using a CARLZEISS colorimeter. Standard curve was prepared using chlorogenic acid and all the concentrations were in terms of mg of these compounds.

2.2.1.3. n Total Soluble Sugars

An aliquot of 100 mg dry powder of leaf material from the shoot system was taken and few drops of cold 80 per cent ethanol and 0.5 ml distilled water were added to prevent clumping. 5 ml of warm 80 per cent ethanol was added to each tube and stirred well to extract the total soluble sugars (McCready **et al.**, 1950). The samples were spinned at 10,000 r.p.m. for 15 minutes. The supernatant was collected and the residue was repeatedly extracted four times with warm ethanol. All the supernatants were pooled together and evaporated to dryness at 40°C. The residue was dissolved in a small quantity of warm water and the final volume was made upto 5 ml. The content of total soluble sugars was determined using Anthrone method (Yemm and Willis, 1954).

2.2.1.3. o Starch

The residue obtained after the ethanolic extraction of sugar was used for further extraction of starch with the addition of 52 per cent perchloric acid and stirred continuously for 5 minutes and occasionally thereafter for 20 minutes. The sample was then spinned at 6,000 r.p.m. for 15 minutes and the supernatant was collected. The residue was extracted again with perchloric acid and the volume of the pooled supernatant was diluted to 100 ml. The supernatant was

dry filtered and the residue was discarded. 10 ml of the perchloric acid extract was hydrolysed with 10 ml of 1 N HCl for 3 hours in a boiling water bath. It was then neutralised with 2 N sodium carbonate and the volume was diluted to 25 ml. An aliquot of this extract was used for the estimation of its reducing sugar content following the method of Folin and Malmrose (Umbreit **et al**., 1959). The estimated glucose content was then converted into starch equivalent by multiplying the glucose value with 0.9.

2.2.2 II Year Study

Based on the results of the first year study it was considered to test the yield pattern of GR 3 rice cultivar in the Kharif season (July-November) 1986, as it showed betterment under the test site. It was also considered to use the harvested seeds of GR 3 cultivar (grown under water normal condition with high nitrogen management) from the test site of I year study, to raise the progeny of the plant exposed to chronic air pollution to verify its tendency to tolerate chronic air pollution for a subsequent generation. To prevent the formation of unfilled grains additional strategic attempts were made with foliar application of polyamines - putrescine, spermidine and spermine, kinetin and ascorbic acid, as an innovative attempt to improve the grain filling rate under the polluted environment.

2.2.2.1 a Growth conditions

Description of the test site, materials, germination of seeds, crop tending practices and air pollution monitoring were as described earlier (1.1.1; 2.2.1.1 b, 2.2.1.1. c, 2.2.1.1 d and 2.1.4. b - f.

2.2.2.1 b Imposition of Regimes

The imposition of the nitrogen regimes was essentially as described in the I year studies (2.2.1.1 e). Progeny plants also had high N treatment with two controls at control site as under :

(i) Progeny of the previous year polluted plants at the test site (PPYPP),
(ii) Progeny of the previous year control plants at control site (PPYCC), and
(iii) Progeny of previous year polluted plants grown at control site (PPYPC).

Treatments as foliar sprays of growth promoters, Kinetin (2 ppm) and Polyamines —10 μ moles of Putrescine, Spermidine and Spermine, and 10 μ moles of antioxidant - Ascorbic acid were carried out at the pre-flowering stage of cultivar, i.e. 45, 47, 49 and 51 day after seeding. The suitable concentrations of these chemicals were decided from their ability to influence growth on the tested treatments at seedling stage (trial - experiment).

2.2.2.2 Growth and Yield Studies

In addition to the morphological characters and yield components studied in the first year the foliar injury, leaf dry weight were determined as growth at 30, 45, 55 and 75 days.

2.2.2.3 Physiological and Biochemical Studies

These studies include Leaf Relative Water Content (RWC) and levels of leaf chlorophylls, phaeophytin, carotenoids, proteins, free proline, ascorbic

acid, polyamines, total nitrogen, sulphur and fluoride.

2.2.2.3 a Relative Water Content (RWC)

At the age of 30 and 55 days fully expanded, third leaf from the top, were randomly sampled and cut into 2-3 cm pieces after weighing (field weight). Then they were kept in petridishes containing 25 ml of distilled water to submerge. After 3 hours all samples were weighed (turgid weight) and kept in an oven at 80°C for 24 hours and again weighed (oven-dry weight). Five replicates were used to calculate the RWC as under (Kramer, 1969) :

$$RWC : \frac{\text{turgid weight - field weight}}{\text{turgid weight - ovendry weight}} \times 100$$

2.2.2.3 b Pigments

For the second year study a recent method described by Laval-Martin (1985) had been used for the determination of Chlorophylls. At the age of 30, 45, 55 and 75 days 200 mg of third leaf from the top were ground in the cold 90% acetone (v/v). This was centrifuged at temperature below 10°C at 3,000 r.p.m., the supernatant was diluted suitably with 90% acetone and the absorption was measured in a CARLZEISS colorimeter. The extraction and assay were carried out in the dark. This method provided controlled Phaeophytinization of chlorophyll present in the extract (addition of 0.1 ml of 3 N HCl to 3 ml of the pigment extract, read after 15 minutes) that permits the measurement of (i) Chl a and b (with a much higher accuracy for Chl b in the special cases in which Chl a/b ratios are greater than 5)and (ii) the total phaeophytin present in the acidified extract. Other than the chlorophyll concentrations comparison between the

molecular concentrations of total phaeophytin and chlorophyll indicates the presence or the absence of initial phaeophytin in the plant material (Laval-Martin, 1985).

Carotenoid pigments were also calculated from the same non-acidified extract measuring at 510 nm and 480 nm as described by Duxbury and Yentsch (1956).

2.2.2.3 c Ascorbic acid

At the age of 30, 45 and 75 day ascorbic acid content was determined from fresh leaf material as described under 2.2.1.3 b.

2.2.2.3 d Protein

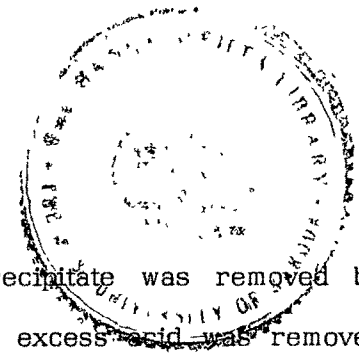
At the age of 40 and 75 days from the fresh leaf materials soluble, insoluble and total protein were determined as described in the I year studies (2.2.1.3 e).

2.2.2.3 e Free Proline

Determination of free proline at the age of 45 and 75 days had been carried out from fresh leaf materials as described under 2.2.1.3 d.

2.2.2.3 f Polyamines

Free polyamines were extracted by grinding one g fresh leaf material



in 0.3 N cold TCA using mortar and pestle. The precipitate was removed by by centrifugation and washed once with 0.3 N TCA. The excess acid was removed by extracting thrice with ether. The extracts were stored in deep-freezer in culture-tubes. Later the extract was chromatographed on a column 0.9 x 15 cm (inside diameter x height) using Amberlite (IRC-50, BDH, K^+ form) as described by Dubin and Rosenthal (1960). Gradient elution was carried out using the method of Busch **et al.**, (1952) with a solution containing 0.1 mole of sodium phosphate buffer, pH 7.2 and 1.65 moles of sodium sulphate per litre. The flow rate was maintained at approximately 20 ml/hour. With same elution conditions, the elution volumes were as follows : for putrescine 90 to 120 ml; spermidine 130 to 167 ml and spermine 210 to 257 ml. The polyamine content of the sample was directly analysed colorimetrically using dinitrofluorobenzene reagent as described by Sanger (1945). An aliquot of the material was diluted to 2 ml with water, then 0.5 ml of saturated sodium borate solution and 0.25 ml of 1.3% dinitrofluorobenzene in acetone were added; after thorough mixing the tubes were kept in water bath at 60°C for 10 minutes. After cooling to room temperature the dinitrophenyl derivatives were extracted with 5 ml of 4 methyl-2-pentanone. The absorption was determined at 420 nm using a Shimadzu 240 model Spectrophotometer. Standards of polyamines were included at each run.

2.2.2.3 g Total nitrogen, sulphur and fluoride

Extraction as well as estimation of foliar nitrogen, sulphur and fluoride content were as described under I year studies 2.2.1.3 h, 2.2.1.3 k and 2.2.1.3 l respectively.

2.3.0 FUMIGATION EXPERIMENTAL STUDIES

2.3.1 Experimental Facilities

The facilities required for air pollution research are both complex and expensive because of the nature of the atmosphere being studied. Though the exposure chambers individually mav be relatively inexpensive, when they are dispensed with automated monitoring portions, they may be highly expensive. Various chamber designs have been recommended for field as well as laboratory conditions. Among the chambers for field exposure most versatile systems are open-top chambers (Heagle **et al.**, 1979) that permit control of the atmospheric composition around the test plants. Runeckles **et al.**, (1978) have fabricated exposure chambers with several filters, fans and other accessories. These chambers have been built to simulate natural conditions to the maximum extent possible, while still permitting control of the pollutant dose.

In the laboratory conditions the gas is introduced inside the chamber and simulated natural conditions are provided. In 1951, Thomas had quoted the investigation of Selby Commissions Report (1915) on the effect of air pollution on plants where artificial fumigation studies were conducted to confirm the field findings. They devised a simple chamber made up of cellophane paper wherein known quantity of SO_2 was injected after diluting it with air. Since then, various chamber designs have been recommended for greenhouse and laboratory (Bell and Clough, 1973; Cowling and Lockyer, 1976; Ashenden and Mansfield, 1977; Ashenden and Williams, 1980) which include enclosed chamber system with air circulation inside the chamber and with wind tunnel fumigation chamber. In India, Banerjee

et al., (1980) have devised a simple fumigation chamber where flow of gas was not continuous. For the present study three glass chambers were used with mini fan facility and inlet, outlet provisions to facilitate an uniform fumigation inside the chamber.

2.3.2 Synthesis of air pollutants

In the present study three gaseous air pollutants namely SO_2, NH_3 and NO_2 were used for fumigation.

2.3.2.1 Sulphur dioxide

To the appropriate amount of sodium bisulphite, dilute sulphuric acid was added to get desired quantity of SO_2 gas. Similar procedure was followed by Rao and Le Blanc (1966) for the production of SO_2.

2.3.2.2 Nitrogen dioxide

Nitrogen dioxide gas was produced by the mixture of sodium nitrite and nitric acid as mentioned by Cotton and Wilkinson (1966). The reaction is as follows :

$$3\ Na\ NO_2 + 2\ HNO_3 \longrightarrow 3\ NaNO_3 + 2\ NO + H_2O \qquad ..(1)$$

$$2\ NO + O_2 \longrightarrow 2\ NO_2\uparrow \qquad ..(2)$$

There were no detection of NO as indicated by brown ring test (Snell and Ettre, 1972).

2.3.2.3 Ammonia

Ammonia gas was synthesized as per the reported procedure of Liptrot, 1983. The involved reaction is as given below :

$$NH_4Cl + NaOH \longrightarrow NaCl + H_2O + NH_3\uparrow$$

2.3.3.0 Fumigation

The fumigation inside the chamber was accomplished by introducing the synthesized gas from the gas sampling flask by a 10 ml gas tight syringe (Hamilton-USA) into the glass chambers through inlet. The mini fans inside the chambers provided the air circulation inside the chamber uniformly equal to a light breeze kept by a regulator.

2.3.4.0 Sampling and Quantification of Air Pollutants

Sampling of air to quantify concentrations pollutants were done 30 minutes after the introduction into the chambers, two times during investigation, for each gas. The air inside the chambers was drawn by vacuum pump through 22 gauge, 1 inch long hypodermic needle through the outlet. The air passed through the absorbing liquid kept in an impinger, at a rate of 1 litre per minute. The number of minutes the sample was run equals to number of litres of air passed through the solution as per Wartburg and Lodge (1968). From the absorbing solution quantification of air pollutants were done. Before removing the pots from the chambers after fumigation the inside air was bubbled through absorbing solutions.

2.3.4.1 Sulphur dioxide

The method followed for the quantification of sulphur dioxide was of Wartburg and Lodge (1968).

Reagents

(i) 0.01 N Sulphuric acid.

(ii) 0.01 N Iodine solution : Carefully weighed 1.269 g of resublimed iodine crystals and 1.5 g of potassium iodide mixed with 2.5 ml of water. The mixture of water, KI and iodine were placed in a mortar and ground thoroughly with pestle until all iodine were dissolved. It was then transferred to a volumetric flask and diluted to 1 litre.

(iii) A starch solution was made by dissolving 1 g soluble starch in 500 ml of cold water. Boiled for few minutes. After cooling it was transferred to a 1 litre volumetric flask. To the flask containing 500 ml of cooled starch 2 ml of 0.01 N H_2SO_4, 8 ml of 0.01 N iodine and 2 g of KI were added. It was then diluted with water to 1 litre and kept in amber bottle away from light and in the impinger while sampling.

It is known empirically that at 20°C and 760 mm Hg, a mole of gas occupies about 24 litres. It was observed by Wartburg and Lodge (1968) that if 10 ml of the absorbing solution were decolourised completely by the sulphur dioxide contained in 9.6 litres of air at 20°C, the concentration of SO_2 would be 1 ppm by volume. Then for example, if 48 litres air caused complete decolourization of 10 ml of reagent, then $9.6/x = 48/1$ or $x = 0.2$ ppm. Accordingly

the calculations were made for the present study of 0.75 ppm of SO_2.

2.3.4.2 Nitrogen dioxide and Ammonia

Absorbing solution and after sampling from the chamber quantification were done as described earlier under 2.1.4. d for NO_2 and 2.1.4. e for NH_3.

2.3.5.0 Culture of Plants

Plants of three cultivars of rice viz., CO 43, GR 3 and TKM 9 were grown in sand culture in plastic pots of 16 cm diameter with nutrient medium as suggested by Yoshida **et al.**, (1976) for rice. Pots were deviced indigenously for this experiments. 'T' shaped glass tubes (9 mm diameter, medium wall) were made, and left arm of the inverted 'T' tubes were inserted at the bottom of the plastic pots and fixed the tube straight parallel to the pot with the help of an adhesive, araldite. The right arm of the inverted 'T' was connected with a rubber tube fitted with a pinch clip. The lengthy arm of the inserted 'T' tube was parallel to the pot used as an indicator to know the level of the medium. The details are depicted in the Plate 4.

2.5 kg/pot of acid washed, uniform sized sand were used to fill the pots after covering the outlet inside the pot with small pebbles, to prevent the blockage of the outlet glass tube by sand while leaching the medium out to wash the sand.

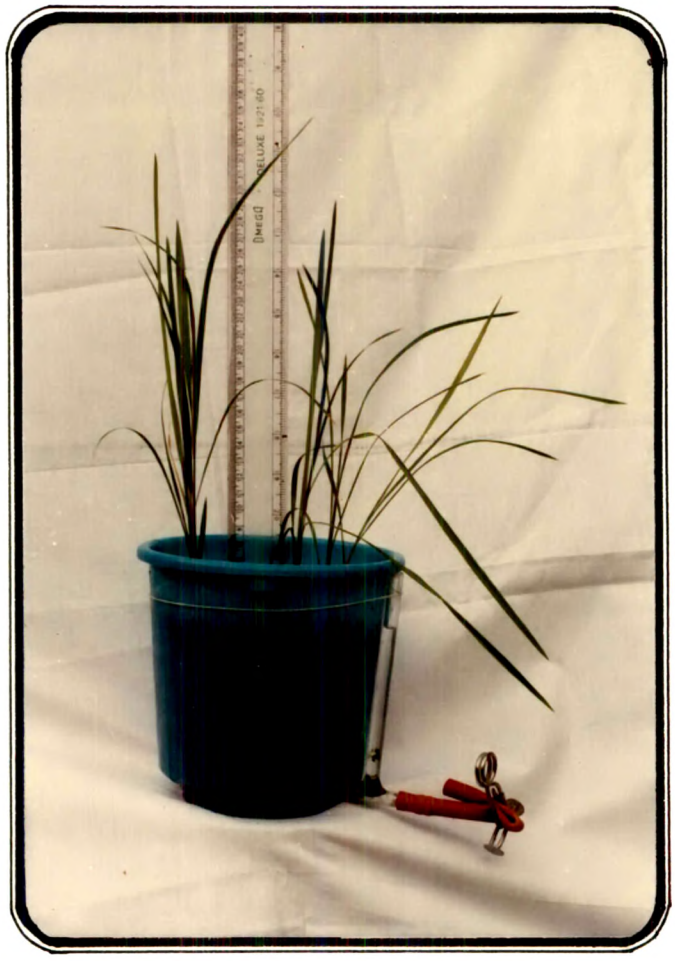


Plate-4 A plastic pot showing rice plants grown in sand-culture for fumigation studies.

2.3.5.1 Medium Composition

Element	Reagent (AR grade)	Concentration (ppm)
N	NH_4NO_3	40.00
P	$NaH_2PO_4.2H_2O$	40.00
K	K_2SO_4	40.00
Ca	$CaCl_2$	40.00
Mg	$MgSO_4$, $7H_2O$	40.00
Mn	$MnCl_2.4H_2O$	0.50
Mo	$(NH_4)_3Mo_7O_2$. $4H_2O$	0.05
B	H_3Bo_3	0.20
Zn	$ZnSO_4$. $7H_2O$	0.01
Cu	$CuSO_4$. $5H_2O$	0.01
Fe	$FeCl_3$. $6H_2O$	2.0

The pH of the culture medium was 5.1. The nitrogen concentration in the medium was doubled to 80 ppm after 3 weeks.

2.3.5.2 Nitrogen regimes

Three levels of nitrogen were maintained in the medium from the day of seeding as 1. Low (20 ppm N), 2. Normal (40 ppm N) and High (80 ppm N). The concentrations of all the three were doubled after 21 days, accordingly.

2.3.5.3 Air Pollution Treatments

21 day-old plants (because active growth of rice plants starts on 20

2.3.6.0 Growth Studies

The growth analysis includes the length and dry weight of shoot and root, total leaf area (TLA), and leaf dry weight. The leaf area per unit leaf weight (specific leaf area, SLA) was also calculated. The data were statistically analysed using an analysis of variance and least significant difference were worked out. For physiological and biochemical analysis standard error of three replicates were worked out except for enzymes.

2.3.7.0 Physiological and Biochemical Studies

2.3.7 a Ascorbic acid

On the third day of fumigations the leaves were taken for the estimation of ascorbic acid as described earlier (2.2.1.3 b).

2.3.7 b Free Proline

On the 4th day after the commencement of fumigations from the leaves the analysis of free proline content was done as described under 2.2.1.3 d.

2.3.7 c Pigments

Five days after the commencement of fumigation treatments 100 mg of leaves were taken for the determination of the chlorophyll 'a', chlorophyll 'b' and carteniods by following the method described earlier (2.2.2.3 b).

2.3.7 d Chlorophyll Temperature Stability Index

The chlorophyll temperature stability index was determined on the 5th day of fumigation treatment from 100 mg of leaves. Leaves were submerged in broad necked tubes containing 10 ml of distilled water for 30 minutes as described by Buxton **et al.,** (1985). Then the leaves were taken out and the pigments were extracted as described earlier. The chlorophyll stability index (mg/g fresh weight of leaves) was calculated as the unheated chlorophyll minus the heated chlorophyll eventually following the procedure of Buxton **et al.,** (1985).

2.3.7 e Total Water Extractable SH Compounds

On the 7th day after fumigation 250 mg of fresh leaves were homogenised with 15 ml of water containing 0.15 per cent ascorbic acid by following the procedure of Grill **et al.,** (1979). The homogenate was centrifuged at 12,000 r.p.m. for 10 minutes. To a 2 ml of the clear supernatant 2 ml of DTNB reagent (3.96 mg 5,5-Dithiobis-nitrobenzoic acid dissolved in 10 ml 0.2 M phosphate buffer, pH 7.0) was added. The yellow colour which developed was measured in 1 cm cuvette at 412 nm in a Shimadzu Spectrophotometer against a blank consisting of 2 ml phosphate buffer giving the absorbance E_1. In a second experiment the absorbance of 2 ml H_2O + 2 ml DTNB reagent was measured against 2 ml H_2O + 2 ml phosphate buffer, giving the absorbance E_2. The SH content of the supernatant (extract) was calculated according to $(E_1-E_2) \times 2 / 13,600$ = mole SH/litre. Finally the SH concentration was expressed as m moles/g fresh weight.

2.3.7 f Glutamine synthetase (EC 6.3.1.2)

One g of fresh shoot portions (8 days after the commencement of fumigation) were homogenized in a mortar and pestle at 0-4°C with 0.1 M phosphate buffer (pH 7.4) containing 10^{-4} EDTA and 10 mM mercaptoethanol. The homogenate was passed through four layers of cheese-cloth and centrifuged at 12,000 r.p.m. for 30 minutes. The supernatant was used for enzyme assay. The extraction procedure was as followed by Sheoran **et al.**, (1981).

The enzyme assay was essentially that of Garg **et al.**, (1985). The reagent mixture of the assay system contained 0.4 ml of 0.2 M Tris HCl buffer (pH 7.5), 0.4 ml of 0.1 M $MgCl_2$, o.4 ml of 2-mercaptoethanol (1:100 v/v), 0.2 ml of 0.5 M Na-glutamate, 0.2 ml of 0.5 M NH_2OH-HCl (1 M NH_2OH-HCl and 1 M NaOH), 0.2 ml of 0.1 M ATP, and 0.3 ml of enzyme extract. The mixture in the tube was shaken well after the addition of each reagent. It was allowed to incubate at 37°C for 15 minutes in a water bath. After 15 minutes, 3 ml of ferric chloride reagent (0.37 M $FeCl_3$, 0.67 N HCl and 0.2 M TCA) was added and allowed for 30 minutes and it was read at 535 nm in a Systronics colorimeter against a reagent blank. A standard using glutamic acid was run with same procedure. The total soluble protein content of the extract was determined by following the procedure of Hartree (1972). The results were expressed in n moles of glutamate released per mg of protein per 15 minutes.

2.3.7 g Nitrate reductase (EC 1.6.6.1)

The extraction and determination of the enzyme activity of the nitrate reductase was from 1 g material on the 8th day of fumigation as described under 2.2.1.3 i .

2.3.7 h Buffering Capacity

On the ninth day of fumigations, one g fresh shoot portion was homogenized in 20 ml distilled water for the determination of the tissue buffering capacity. From the homogenized solutions 10 ml was titrated against 0.05 N HCl and other 10 ml with 0.05 N NaOH to see the pH change. For the present study a digital pH meter itself was used to read the pH differences accurately, leaving the traditional methods of using chemical indicators.

2.3.7 i Leaf Absorbance

For the treatment of mixture of SO_2, NH_3 and NO_2, after 10 days of fumigations leaf absorbance was measured. Fully expanded third leaf from the top was taken for this study. Leaving 2 cms at the top, 3 cms long leaf pieces were cut and fixed in the Integrating Sphere Attachment of Shimadzu Spectrophotometer (model 240). The leaf absorbance were directly recorded at the wavelength between 520 nm and 750 nm, whereunder abosorbance variation was observed evidently among the treatments.

2.3.7 j Total Nitrogen and Sulphur

From the dry shoot portions of 10 days fumigated plants total nitrogen and sulphur were determined as described earlier (2.2.1.3h; 2.2.1.3k). The total nitrogen was estimated for all the treatments, and sulphur content was restricted to treatments where SO_2 was involved.