Chapter 2

MATERIALS & METHODS

CONTENTS

2.1 STUDY AREA

The area under investigation comes under Vadodara Urban Development Authority (VUDA) with a total area of 714.56 Km^2 including 108.00 Km^2 area covered by Baroda Municipal Corporation in the centre and 103 surrounding villages covering the remaining area.

2.1.1 Location

The VUDA area is situated between 78° to 74° 10' E longitude and 21° to 23° N latitude in central Gujarat (western part of India) and is 30 m above mean sea level. The area is on plain level and is traversed by three main rivers and their tributaries, they all flow in the south-west direction. (1) Vishwamitri - It flows through the centre of the city. (2) Mahi - It is 19 Km NW of Baroda and forms the western boundary of the area. (3) Mini - It is a small river which originates from North and after traversing 56 Km. joins Mahi river (Sabnis, 1967).

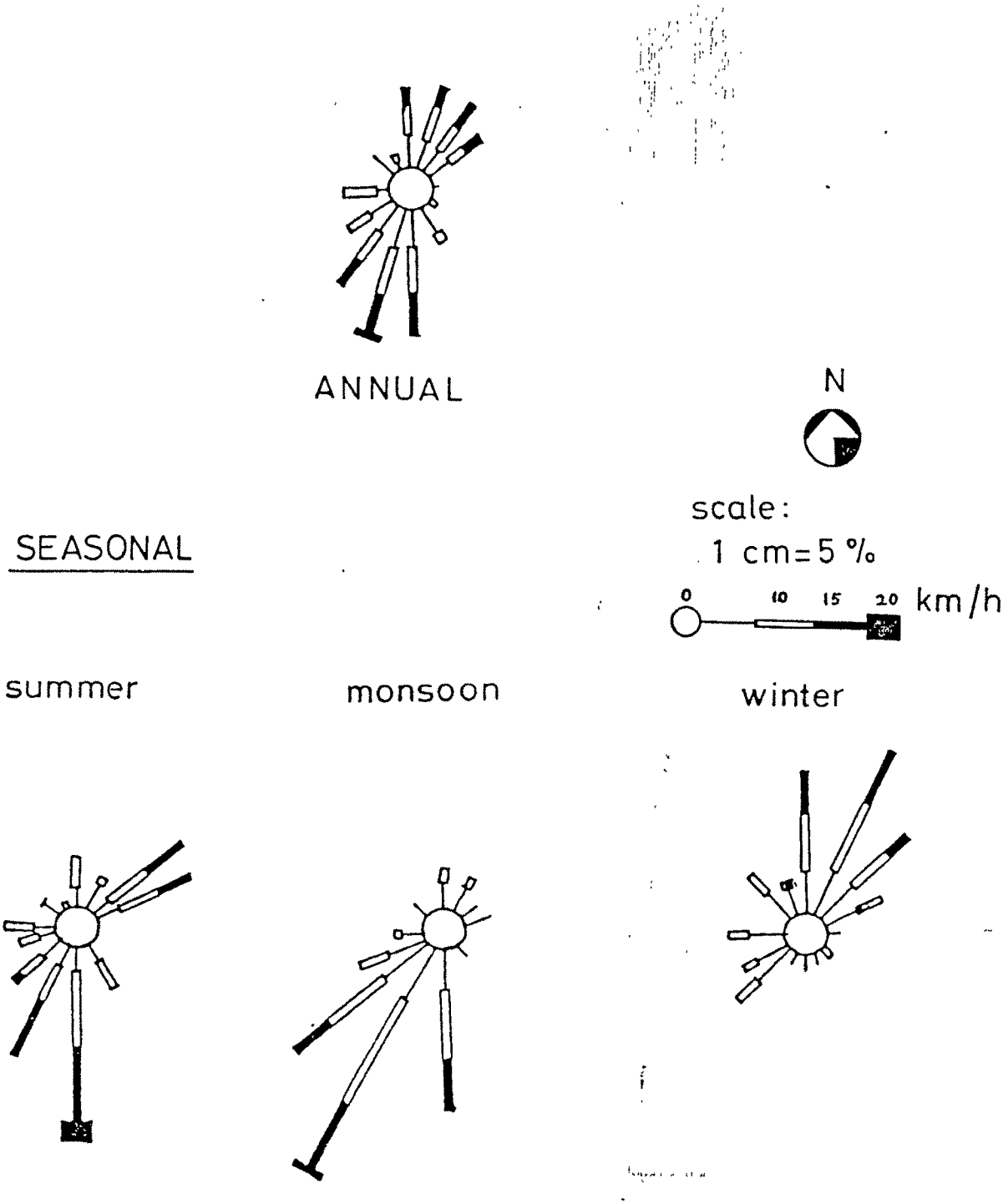
2.1.2 Soil

Soil of the area is black loamy. The broad classification of agricultural productivity of land shows that north western sector consists of highly fertile land. Total cultivated land is 473.20 Km^2 out of which 247.78 Km^2 area is covered by cash crops.

2.1.3 Climate

The year can be divided into three seasons (1) Summer, (March to June) - it is hot and dry period of the year. Wind direction in this season is mostly South-West. (2) Monsoon, (July to October) - it is rainy season, climate is hot and humid. Wind direction in this season is also South-West. (3) Winter, (November to February) - it is comparatively pleasant period of the year. Generally it is cold and dry period. The prevailing wind direction is North-East. Meteorological parameters such as minimum and maximum temperature, relative humidity, wind speed and wind direction were obtained from the Meteorological observatory, M.S. University, Baroda, for four years during the study period (1983-1987). Windrose diagrams were prepared from data of wind speed and direction (Fig.1). Temperature and Humidity are given in Table 3.

Fig. 1 : WINDROSE DIAGRAMS



2.1.4 Pollutants & Their Sources.

The industrial development of study area has been given (1.7.4). Present investigation was conducted at the North-West region of VUDA area. The main industries situated in this area are an oil refinery, a petrochemical complex, a fertilizer complex, an alkalies and chemicals plant, a phenolics unit and an industrial estate comprising 369 small and medium-scale chemical industries in an area of 5 Km^2. Products and pollutants of these industries are given in Table 4. The National High-way No.8 with a heavy vehicular traffic also passes through the study area and auto-exhaust pollution by this adds to the damage caused by industrial pollutants.

2.2 SELECTION OF ZONES

Major industries and pollutants of the area under investigation have been given in Table - 4. National Institute of Occupational Health (NIOH) monitored ambient air concentration of major gaseous pollutants (SO_2 & NO_x) at different places (1982-1986) in the study area under a project sponsored by VUDA.

An initial survey was done to record the vegetation damage at various places. Visual observations and information from local authorities were collected. A few zones were selected on the basis of this survey and monitoring data taken from VUDA. The selected zones were classified as high, medium and low pollution zones. Two zones with lesser air pollution were also selected away from the major industries. These zones were referred as reference zones R_1 & R_2 (Fig.2) .The number of zones were arranged serially in the order of increasing concentration of pollutants.

Zone No.	Pollution Status
I, II, III	Low
IV	Medium
V, VI, VII, VIII	High

2.3 MONITORING OF MAJOR AIR POLLUTANTS AT SELECTED ZONES

Ambient air concentration data for major air pollutants was taken from VUDA reports. Monitoring at each zone was done on every 6th day. From these observations monthly and seasonal average concentrations and range of minimum to maximum concentration during the year were also recorded. Some of the peak concentrations due to accidental leakage, shut down and start up or other reasons (which might have occured when monitoring was not being done) could not be recorded in absence of continuous monitoring system.

Location of monitoring points for respective zone has been given in Fig.2. Monitoring point 5 was lying between zone IV & V. Zones VII & VIII also had a common monitoring point 7 but its location was more nearer to zone VII (0.25Km), zone VIII was 0.75 Km away from the monitoring point (Fig 2). Common monitoring was done due to lack of facilities.

High volume sampler was used for monitoring of the pollutants. Air was continuously passed through impingers having absorbing media for a particular gas. Three air samples each for the duration of 8 hours were collected integrating for 24 hours.

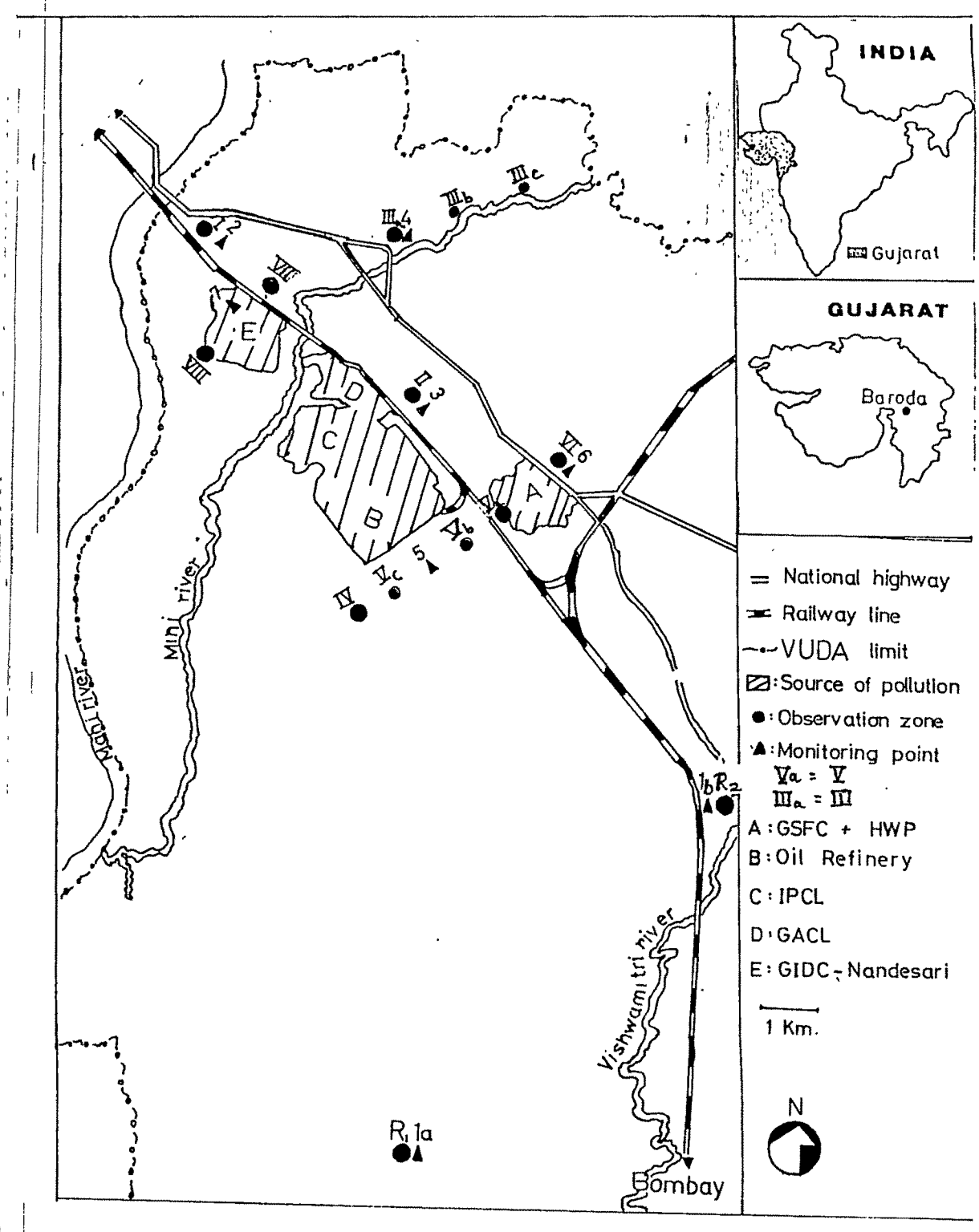
SO_2 was monitored by West & Gaeke Method (1956). The gas was absorbed in potasium tetrachloromercurate (TCM). The solution was treated with sulphamic acid and formaldehyde. Pararosaniline reagent was used to develop the colour. Optical density of the treated samples was measured at 560 nm. SO_2 concentration thus calculated in ppm was converted to μgm^{-3} by the following formula:

$$\underset{\mu gm^{-3}}{SO_2} = \frac{ppm \times 64 \times 10^6}{24470}$$

For preparing standard graph sodium metabisulphite was used.

NO_x monitoring was done, following the method described in Indian Standard Institute 5182 Part VI (1975). The gas was absorbed in 0.1N sodium hydroxide solution. Solution was then treated with hydrogen peroxide, sulphanilamide and N-ethylene diamine dihydrochloride. The colour developed was read at 540 nm. NOx concentration in µg was obtained from standard graph prepared with sodium nitrite. Monitoring of chlorine was done at zone II and VIII. Free chlorine was absorbed in the alkaline solution (NaOH) and the estimation was done by colorimetric, o-tolidine method (Rand *et al.*, 1977).

Fig. 2 : MAP OF THE STUDY AREA



2.4 DESIGNING OF EXPERIMENTS

2.4.1 Field Survey

Survey of field grown crops was done at different zones, at different distances and directions from major sources of pollution, in different seasons. Assessment of damage was done by visual observations made at different times during the life cycle of crops. Observations were recorded from random sampling ($1m^2$) in fields. Average of twenty five samples for each crop at each zone was taken.

Detailed survey :

Following crops were selected for this survey :

1. *Nicotiana tabacum* Linn. Var. A-119
(Eng. Tobacco; Vern. Tambacu)

2. *Solanum tuberosum* Linn. Var. Kufri
(Eng. Potato; Vern. Alu, Batata)

3. *Cajanus cajan* Spreng Var. BDN-2
(Eng. Pigeon pea; Vern. Arhar, Tur)

Survey was conducted at all the eight polluted zones and reference zone (R_1). At each zone ten crop fields were selected for each species. Representative sampling in the fields was done. Observations were recorded periodically (at 30 days interval) till crop maturity. Parameters studied were plant height, total leaf area, visible symptoms, % leaf area damage, above ground biomass, relative growth rate (RGR) and net assimilation rate (NAR). Yield was recorded at harvest. Sampling method was same as in previous survey.

2.4.2 Potted Plant Exposure Study

Three major solanaceous species were selected for this study.

1. *Nicotiana tabacum* Linn. Var A-119
(Eng. Tobacco; Vern. Tambacu)

2. *Solanum tuberosum* Linn. Var. Kufri
(Eng. Potato; Vern. Alu. Batata)

3.*Solanum melongena* Linn. Var. S-16 round
(Eng. Brinjal, Egg plant. Vern. Bengan, Ringan)

Perforated polythene bags (35 x 30 cm), having garden soil rich in organic manure were transferred to different zones. Ten pots for each crop were kept at each zone (I-VIII and R_2) in wirenet fenced cages.

Twenty days old tobacco and egg plant saplings were transplanted in the pots. Potato eyes were sown directly in the bags. Cultural practices were uniformly maintained for all the pots, at all the zones. Pots were equally watered from a common source (university campus) at regular interval. Observations for each parameter were recorded at regular interval of 20 days upto maturity.

Morphological and growth parameters studied were height, number of leaves, total leaf area, % leaf area damaged (injury index), above ground biomass, relative growth rate (RGR) and net assimilation rate (NAR). Foliar epidermal features were studied once in the life cycle.

Biochemical parameters like chlorophyll, soluble sugars, sulphur, nitrogen and chloride content in the foliar tissue were estimated. At maturity of the crop nicotine content of tobacco leaves and starch content of the potato tubers was estimated. Yield was recorded at the harvest.

2.4.3 Artificial Fumigation Study

The study was conducted under simulated conditions. Sulphur dioxide gas was selected to study its effects. Fumigation was done in an acrylic chamber (3 m^3). The chamber received air at a rate sufficient to provide one air change per minute. A metered quantity of SO_2 from a cylinder was added to one of the air streams to achieve the desired SO_2 concentration (0.2 ppm). Concentration inside the chamber was monitored during the exposure period (West & Gaeke, 1956). During exposure time, temperature inside the chamber was more by 1°C and ± 5% change in relative humidity was seen as compared to ambient conditions.

Twenty days old saplings of tobacco were transplanted in perforated polythene bags (30 x 35 cm) filled with humus rich soil. Two sets each of 5 pots were prepared for the experiment. One set was exposed to sulphur dioxide while one set was kept unexposed and was taken as reference. Exposure was done for 2 hours on alternate days starting from thirty days old plants till 120 days age of plants. Reference plants were also kept for 2 hours inside the chamber without any artificial gas exposure.

Code

E = exposed to SO_2
UE = unexposed to SO_2

Observations were recorded at regular interval of 20 days. Morphological parameters studied were height, number of leaves, total leaf area and % leaf area damage. Biomass was also recorded. RGR and NAR were calculated for growth performance. The biochemical parameters recorded were chlorophyll, protein, soluble sugars, ascorbic acid and sulphur content of foliar tissue. At harvest yield was recorded.

2.4.4 Mitigation of Pollution Damage

a. Under simulated conditions

Experiment was carried out on pot grown tobacco crop. Eight sets, each of 5 pots were prepared. Three concentrations of ascorbic acid aqueous solutions were selected for the study viz. 0.005, 0.0075 and 0.01 M (Molar). Two sets for each concentration were taken. The respective solutions were foliarly sprayed on the plants of the six sets. Five sprays at regular

interval of 20 days were given starting with the initial spray on the 20 days and final on 100 days old plants. One set from each treatment was exposed for two hours on alternate days to 0.2 ppm sulphur dioxide. The fumigation was initiated on 20 days old plants and was carried out upto 120 days. The remaining two sets were kept as untreated sets, one set was exposed to 0.2 ppm SO_2 the other was kept as unexposed.

Observations were recorded at regular interval of 20 days. Morphological parameters viz. height, number of leaves, total leaf area and % leaf area damage were studied. Above ground biomass was also recorded. Growth analysis was done for RGR and NAR. Biochemical parameters like chlorophyll, protein, ascorbic acid, soluble sugars and sulphur content of leaves were studied. Yield was recorded at harvest.

Percentage recovery in different parameters in treated and exposed plants (T_1E, T_2E & T_3E) were calculated with reference to untreated and exposed plants (UTE). Treated and unexposed plants (T_1, T_2 and T_3) were used to know the difference from normal plants (UTUE).

Code for treatment:

Exposed	Unexposed	Chemical concentration	Amount sprayed
T_1E	T_1	0.005 M Ascorbic acid	0.18 gm m^{-2}
T_2E	T_2	0.0075 M Ascorbic acid	0.27 gm m^{-2}
T_3E	T_3	0.01 M Ascorbic acid	0.36 gm m^{-2}

UTE = Untreated and exposed
UTUE = Untreated and unexposed.

b. Under field conditions

To south west of a fertilizer complex (zone V, Fig. 2) three fields were selected at a distance of 0.5 -0.6 Km having tobacco, potato and egg plant crops grown by farmers. Tobacco

and egg plant were transplanted in monsoon (September) while potato was planted in winter (November). All the three crops were harvested in early summer (March). The selected fields were in leeward direction during monsoon and in windward direction during winter.

In each field 21 plots of $1m^2$ were made. Three plots per treatment were kept. 3 plots were kept untreated and were used as reference. Aqueous solutions of the following concentrations of the two chemicals were used for the abatement study.

Urea : 0.15 M, 0.30 M, 0.45 M

Ascorbic acid : 0.005 M, 0.01 M, 0.02 M

Foliar spray of the solutions of different concentrations, was done on respective sets of the three crops. Reference plants were sprayed with distilled water.

Observations were recorded at regular interval of 20 days. Morphological parameters recorded were height, number of leaves, total leaf area and % leaf area damage. Above ground biomass, RGR and NAR were also recorded. Biochemical analysis of foliar tissue was done for chlorophylls, protein, ascorbic acid, soluble sugars and sulphur content. Yield was recorded at harvest. Percentage recovery for each parameter was calculated with respect to reference (untreated) plants. Cost benefit ratio was calculated from the yield data.

Code for treatment :

Code	Chemical concentration	Amount sprayed
U_1	0.15 M Urea	1.80 gm m^{-2}
U_2	0.30 M Urea	3.60 gm m^{-2}
U_3	0.45 M Urea	5.40 gm m^{-2}
A_1	0.005 M Ascorbic acid	0.18 gm m^{-2}
A_2	0.01 M Ascorbic acid	0.36 gm m^{-2}
A_3	0.02 M Ascorbic acid	0.72 gm m^{-2}
C	Untreated with any chemical	-

2.5 METHODS USED FOR DATA COLLECTION OF VARIOUS PARAMETERS

2.5.1 Morphological & Growth Parameters

Different parameters studied were expressed as shoot length (cm/plant), number of leaves (No./plant), total leaf area (cm^2/plant). Total leaf area and damaged leaf area were calculated with graph paper and the percentage leaf area damage (injury index) was calculated by the following formula

$$\text{Injury index (\%)} = \frac{\text{Damaged leaf area} \times 100}{\text{Total leaf area}}$$

Injury index was not recorded at maturity stage (last observation in all experiments), because it was difficult to distinguish the injury symptoms from normal maturity symptoms. Biomass was recorded on dry weight basis (of above ground vegetative parts) by oven drying the samples and was expressed as gm/plant dry wt.

Growth analysis for relative growth rate (RGR) and net assimilation rate (NAR) was done by following formulae (Evans, 1972).

$$\text{RGR (mg./gm./day)} = \frac{1}{W} \cdot \frac{dW}{dt} = \frac{\ln W_2 - \ln W_1}{T_2 - T_1}$$

$$\text{NAR (mg./cm}^2\text{/day)} = \frac{1}{F} \cdot \frac{dW}{dt} = \frac{(W_2 - W_1)(\ln F_2 - \ln F_1)}{T_2 - T_1}$$

where W_1 = Initial dry weight of plant
W_2 = Final dry weight of plant
F_1 = Initial leaf area
F_2 = Final leaf area
T_1 = Time of initial observation
T_2 = Time of final observation
W = Increase in dry weight ($W_2 - W_1$)
F = Increase in leaf area ($F_2 - F_1$)
dW/dt = Slope of total dry weight curve over time.

Methods adopted for recording yield were different for different crops. Tobacco yield was recorded by taking dry weight of leaves at maturity and was expressed as Kg 100^{-1} m^2 dry wt (air dried). Potato yield was recorded by weighing freshly harvested tubers and was expressed as Kg 100^{-1} m^2 fresh wt. In egg plant the fruit production was gradual. For recording yield, total number of fruits per plant were multiplied by average fresh weight of fruit and was expressed as Kg 100^{-1} m^2 fresh weight of fruits. Percentage flowering and fruiting were also recorded. Average number of flowers and fruits per plant, counted at reference zone, was taken as 100% and values at other zones were recorded with respect to reference.

Foliar epidermal study

Leaves of tobacco, egg plant (at 80 days age of plants) and potato (at 60 days age of plants) were collected from high pollution zone (V) and reference zone (R_2). Collected leaves were immediately fixed in FAA (Formaline 40%, Glacial Acetic acid and Alcohol 50%; 5:5:90) and brought to laboratory. Epidermal peels were taken manually and after staining in saffranine were observed under microscope. Number of epidermal cells, trichomes and stomata per unit area were counted, stomatal index was calculated by the following formula:

$$\text{Stomatal Index} = \frac{S \times 100}{S + E}$$

Where

S = No. of stomata per unit area

E = No. of epidermal cells per unit area.

2.5.2 Biochemical Parameters

Foliar analysis was done for different biochemical parameters viz. chlorophyll ascorbic acid, proteins, total soluble sugars, sulphur, chloride, nitrogen and nicotine content. Leaf samples were collected, washed under running tap water and were subjected to analyses in fresh or dried condition according to the procedure. Starch content in dried potato tubers was also determined.

2.5.2.1 Chlorophyll content

The extraction of chlorophyll was done by Holden's (1965) method. 100 mg of fresh leaf sample was crushed in a pre-chilled mortar and pestle with 80% cold acetone. A pinch of calcium carbonate was added to prevent chlorophyll degradation during estimation. Glass powder was added for easy maceration. The extract was centrifuged at 3000 rpm for 15 minutes. The supernetant was collected. The process was repeated till the residue became colourless. The collected supernetant was diluted upto required constant volume. The samples were kept in dark to avoid chlorophyll degradation. Optical density of the extract with reference to blank was measured at different wavelengths.

Chlorophyll *a* & *b* contents were determined by Maclachlan and Zalik's (1963) formulae:

$$\text{Chlorophyll } a \text{ mg/gm fresh wt.} = \frac{(12.3 \times OD_{663}) - (0.86 \times OD_{645}) \times V}{d \times 1000 \times w}$$

$$\text{Chlorophyll } b \text{ mg/gm fresh wt.} = \frac{(19.3 \times OD_{645}) - (3.6 \times OD_{663}) \times V}{d \times 1000 \times w}$$

Where V = Total volume of supernetant (ml)
d = Distance travelled by light (cm)
w = Weight of the sample (gms)
OD = Optical density at particular wavelength

2.5.2.2 Ascorbic acid content

It was estimated by Schaffert and Kingsley's (1955) method. 100 mg of fresh leaf sample was ground with 0.5% oxalic acid and centrifuged at 3000 rpm for five minutes. Supernetant was taken and definite volume was made. To this activated charcoal was added for conversion of ascorbic acid into dehydroascorbic acid. 2,4- Dinitro phenyl hydrazine was used for colour development which was measured at 515 nm. The total ascorbic acid content was calculated from the standard graph, plotted for AR ascorbic acid.

2.5.2.3 Protein content

It was determined by Hartree's (1972) method. 100 mg fresh leaf sample was ground in 0.05N NaOH. Folin & Ciocalteu reagent was used for colour development. The optical density was measured at 650 nm. The standard graph was plotted with crystalline Bovine albumin.

2.5.2.4 Sugars

The extraction was done by the method of Mc Cready *et al.*, (1950). To 100 mg dry leaf sample boiling ethanol and water (1:1 v/v) were added. After centrifugation at 5000 rpm for 15 minutes, the supernetant was collected. Process was repeated for five times. Collected supernetant was evaporated and dissolved in water, estimation of total soluble sugar was done from this. Residue left in extraction was used for starch estimation.

i. Soluble sugar

The estimation was done by Yemn and Will's (1954) method, with anthrone reagent. The colour developed was read at 620 nm. The standard graph was prepared with glucose.

ii. Starch

Estimation was done by Mc Cready *et al.*, method (1950). Residue left after extraction (as mentioned above) was dissolved in perchloric acid and then estimation was done for soluble sugars. The content thus obtained was multiplied with 0.9 to get starch content.

2.5.2.5 Sulphur content

It was determined by Garrido's method (1964). The dried and powdered leaf sample (100 mg) was digested with mixture of nitric acid and perchloric acid (3:2). The turbidity was determined at 420 nm after addition of Barium chloride-tween 80 reagent. Standard graph was plotted with ammonium sulphate.

2.5.2.6 Chloride content

Its estimation was done by Humphrie's (1956) method. Dry leaf samples were fused with calcium oxide and then ashed at 500°C. Double titration with silver nitrate and standard

ammonium thiocyanate was done to estimate chloride content, using the following formula.

1 ml. of 0.1 N $AgNO_3$ = 3.55 mg. chlorine.

2.5.2.7 Nitrogen content

It was determined by Miller and Miller's method (1948). Dry leaf powder was digested with sulphuric acid and hydrogen peroxide. The digested samples were diluted to a definite volume. Nessler's reagent was used for colour development which was read at 420 nm. Ammonium sulphate was used for preparing standard graph.

2.5.2.8 Nicotine content

It was determined by Willits *et al.*, method (1950). The dried and powdered leaf samples were mixed with magnesium oxide (1:1 w/w). The mixture was steam distilled. The distillate was collected in 0.05 N HCl kept in ice bath. The UV absorbance of distillate was measured at 236, 259 and 282 nm against blank. Nicotine content was calculated by the following formula.

$$D'_{259} = 1.059 \{FD_{259} - 1/2 (D_{236} + D_{282})\}$$

$$C = D'_{259}/(34.3x\ b)$$

Where

D'_{259} = Density correction for background absorption
D_{236} = Observed density at 236 nm
D_{282} = Observed density at 282 nm
FD_{259} = Observed density of diluted solution (dilution factor is 10) at 259 nm.
C = Nicotine content gms/litre
b = Inside depth of cell = 1 cm.