Chapter II

# MATERIALS & METHODS

# MATERIALS AND METHODS

The location and climate of Baroda [illegible] (VUDA area), major industries, their location, main products and pollutants have been described in the Chapter 1.1.4.

## 2.1. SURVEY OF GENERAL VEGETATION NEAR A FERTILIZER COMPLEX

Gujarat State Fertilizer Complex located at 8 Km. north-west of Baroda City was selected as pollution source for this study. As the area under study was a disturbed area and cultivated land, quadrat study was conducted on the herbaceous species of natural vegetation in open spaces only. A belt of 2.5 Km. length was taken on the windward direction. Quadrat study was conducted [illegible] the belt (East-west) at a distance of 0.5, 1.5 and 2.5 Km., away from the source. Size and number of quadrats were determined by standard ecological methods (Misra, 1968). The species frequency and number of species were recorded in 30 quadrates of 1 x $1m^2$ size, at each point i.e. at 0.5, 1.5 and 2.5 Km. distance away from the source of pollution. Four classes were made based on the percentage of frequency:

| | | |
|---|---|---|
| Less than 25% frequency | = | Rare (R) |
| 25 to 50% frequency | = | Less common (LC) |
| 50 to 75% frequency | = | Common (C) |
| 75 to 100% frequency | = | Very common (VC) |

Careful visual observations were recorded for the damaging symptoms exhibited by the trees, shrubs and herbs. Symptoms formed due to aging or factors other than pollution were differentiated and recorded. The degree of visual damage was observed based on the percentage leaf area damaged or leaflessness. The plants were grouped in four categories based on degree of damage viz.,

| | | |
|---|---|---|
| No visible damage | = | Nil |
| Below 20% damage | = | Less damaged (LD) |
| 20 to 50% damage | = | Damaged (D) |
| Above 50% damage | = | Highly damaged (HD). |

## 2.2. STUDY ON THE TREES GROWING ALONG THE NATIONAL HIGHWAY NO.8 - A GRADIENT ANALYSIS

The combined effect of industrial air pollution and autoexhaust on Dalbergia sissoo Roxb. (sisham) an economically important timber species and Syzygium cumini Skeels (jamun) a fruit tree growing along the National Highway No. 8 was studied. The pollution impact on growth parameters like height of the tree, canopy cover, circumference at breast height and leaflessness were investigated. This study was conducted during monsoon season to avoid the natural leaf shedding period.

### 2.2.1. Study area:

The area under investigation was on Bombay - Ahmedabad National Highway No. 8 passing through Baroda. Here

vehicular traphic per day was 15000 to 16000. A belt of 6 Km. length from Baroda Municipal Corporation (BMC) limit upto Dashrath village was taken for the study. The belt was divided into seven sectors (Fig.2.1.). Each sector was 0.8 to 1.2 Km. long. In sector 4 and 5 are located a fertilizer complex (GSFC) and Heavy Water Plant (HWP).

Sectors:

1. Baroda Municipal Corporation limit to Zenith Tin Works.
2. Zenith Tin Works to Chhani Bus stop.
3. Chhani Bus stop to Railway bridge.
4. Railway bridge to Writon.
5. Writon to Fertilizernagar gate.
6. 0.8 Km., from Fertilizernagar gate towards Dashrath.
7. Upto Dashrath Village (Fig.2.2.1.).

2.2.2. Morphological observations:

Detailed description on measurement of height, CBH, canopy cover and leaflessness are described in next Chapter 2.3.1. Field survey of selected fruit trees. Initial plantation data was obtained from State Forest Department. (Syzygium cumini trees were planted in 1946, whereas Dalbergia sissoo were planted during, 1974).

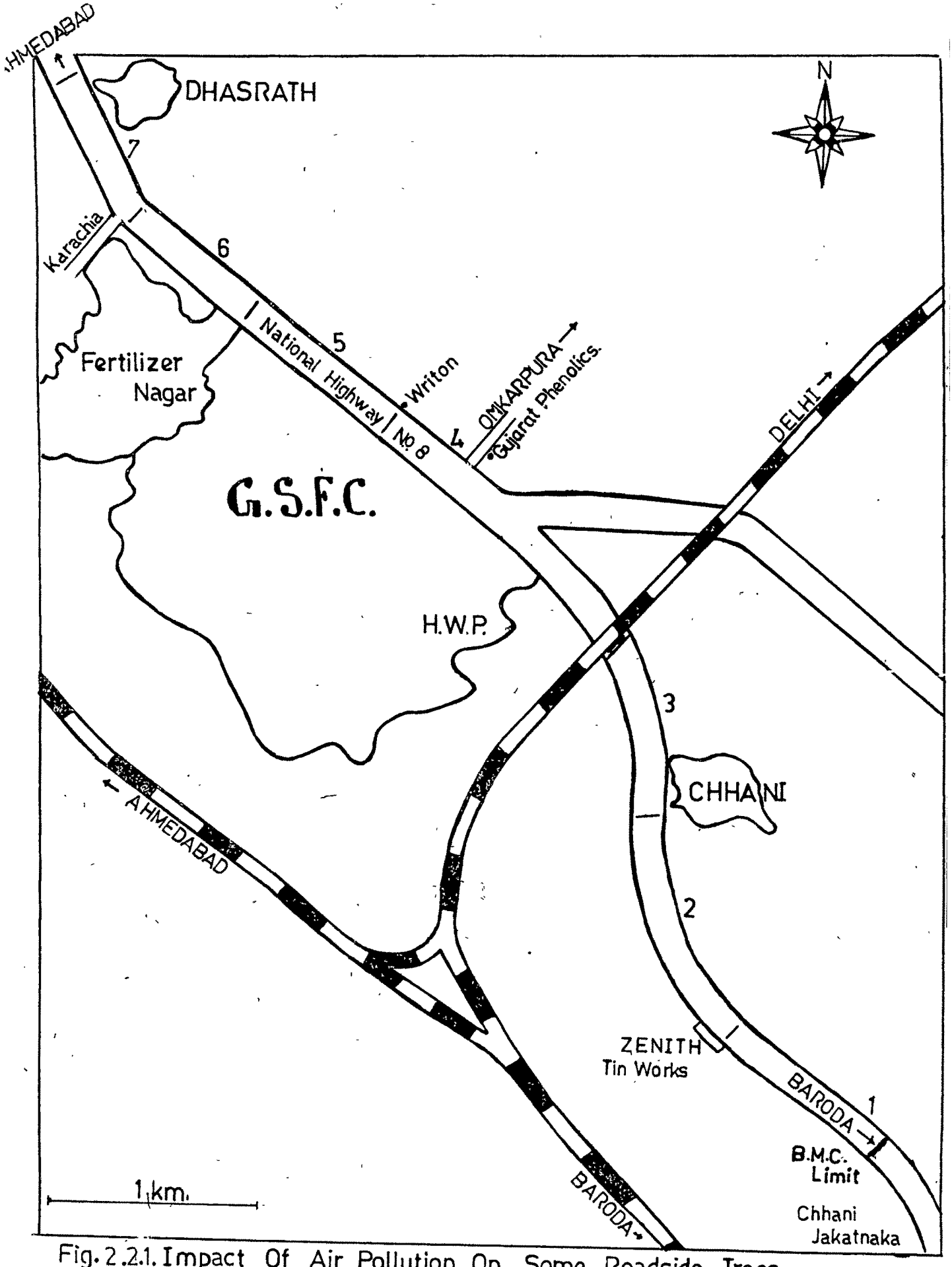



Fig. 2.2.1. Impact Of Air Pollution On Some Roadside Trees.
(Figure showing sectors 1 to 7)

Plantation data was available only for Dalbergia sissoo Roxb. (Table 10). Comparisions were made between density of trees exist in each sector and number of trees planted. For density study, quadrates of 30 x 8 mts., were laid.

Different classes were made for each parameters and percentage of trees representing each class were determined (Table 4 and 5). This was to avoid the variations in the length of the sector and variable number of trees in each sector. The tree casuality like dead, dried, cut down and removed have not been considered for calculating percentage trees in each sectors. (Table 10).

Table 4. Different classes for Dalbergia sissoo Roxb.

| Parameter | CLASS | | | | | | |
|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G |
| Height (mts.) | Below 5 | 5 to 6 | 6 to 7 | 7 to 8 | 8 to 9 | 9 to 10 | Above |
| CBH (cms) | Below 30 | 30 to 45 | 45 to 60 | 60 to 75 | 75 to 90 | Above | .. |
| Canopy cover ($m^2$) | Below 15 | 15 to 20 | 20 to 25 | 25 to 30 | 30 to 35 | 35 to 50 | Above 50 |

Table 5. Different classes for Syzygium cumini Skeels

| Parameter | CLASS | | | | | | |
|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G |
| Height (mts) | Below 6 | 6 to 7 | 7 to 8 | 8 to 9 | 9 to 10 | Above 10 | - |
| CBH (cms) | Below 100 | 100 to 120 | 120 to 140 | 140 to 160 | 160 to 180 | Above 180 | - |
| Canopy cover ($m^2$) | Below 50 | 50 to 75 | 75 to 100 | 100 to 125 | 125 to 150 | 150 to 175 | Above 175 |

2.3 STUDY ON SELECTED FRUIT TREE SPECIES

Detailed investigation was carried out on three economically important tropical fruit trees viz.,

Mangifera indica L. (Eng. Mango, vern. Keri, Ambo: Family Anacardiaceae)

Manilkara hexandra (Roxb.) Dubard. Syn. Mimusops hexandra Roxb. (vern. Rayan, Kirni; Family Sapotaceaee)

Syzygium cumini Skeels Syn. Myrtus cumini L., Eugenia jambolana Lam. (Eng. Jamun; Vern. Jambu, Jambhal: Family Myrtaceae)

which are common species around the aforesaid industrial complexes.

This study was conducted in three phases:

(i) Field survey: The extent of damage by air pollution on fruit trees growing in natural condition at different villages

was investigated. The extent of damage was recorded on morphological yield and biochemical parameters by comparing with the control observations.

(ii) Field exposure study: Exposure of experimental potted plants (mango, rayan, and jamun saplings) to ambient air at different stations in the pollution zone.

(iii) Artificial fumigation study: Experimental potted plants (mango, rayan and jamun saplings) were exposed to known concentration of sulphur dioxide under laboratory condition.

During this study period, National Institute of Occupational Health (NIOH) was monitoring ambient air quality for VUDA at most of the observation stations. This was considered as one of the main factors while selecting the observation stations for field survey and field exposure study. Some stations where ambient air monitoring was not carried out, were also considered for the study.

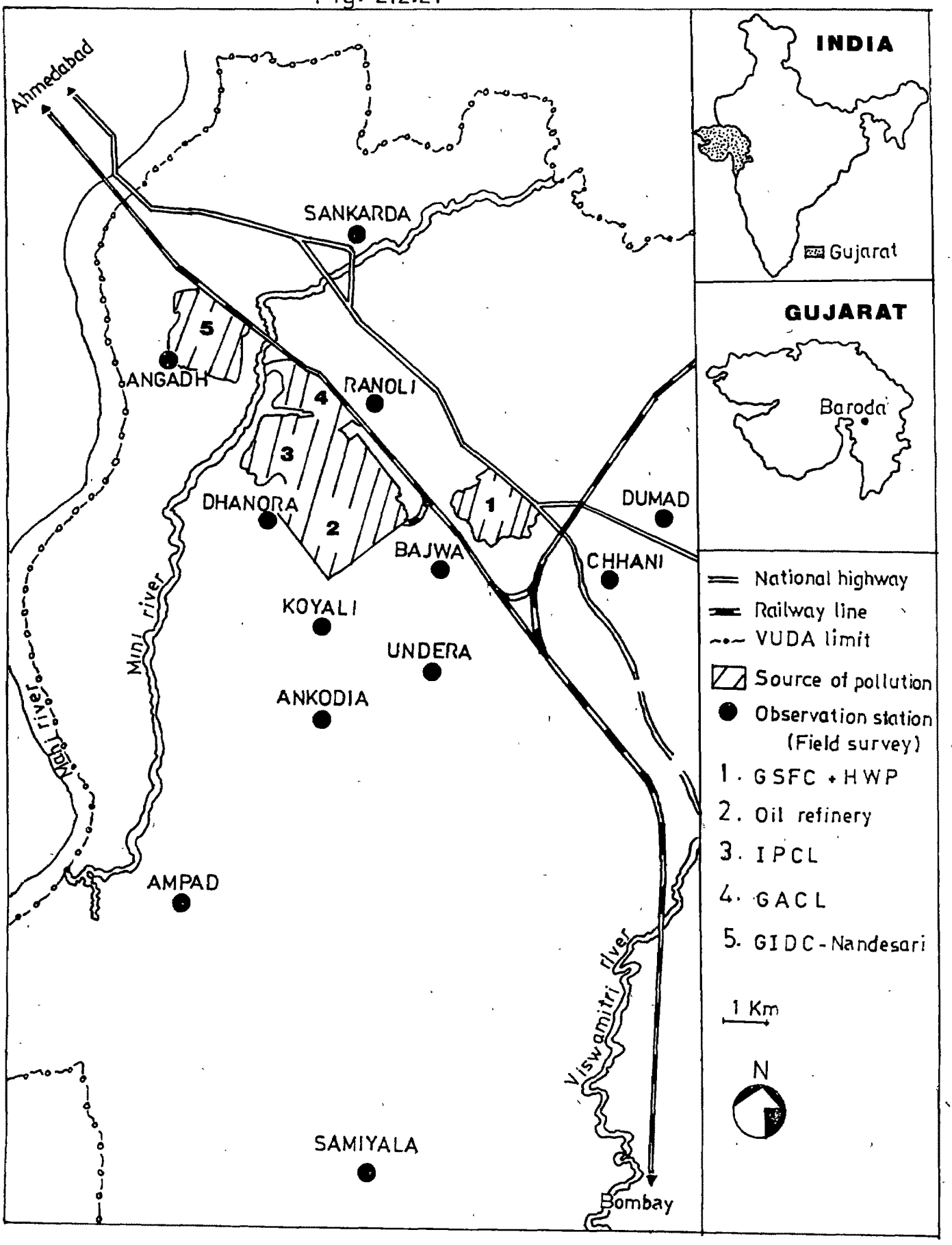
2.3.1. Field Survey:

(i) Selection of site: The extent of damage on different morphological, yield and biochemical parameters of selected fruit tree species was investigated at the following eleven villages: Four villages viz., Bajwa, Angadh, Ranoli and Dhanora were selected from high pollution zone i.e. stations located in very close vicinity (0.5 to 1 Km., distance)

Fig.2.2.2. Map showing the location of sources of pollution and observation stations for field survey

1. Gujarat State Fertilizer Company and Heavy Water plant
2. Gujarat oil refinery
3. Indian Petrochemicals
4. Gujarat Alkalies and Chemicals Ltd.
5. Nandesari Industrial Estate

Fig. 2.2.2.


Ahmedabad
SANKARDA
ANGADH
RANOLI
DHANORA
BAJWA
DUMAD
CHHANI
KOYALI
UNDERA
ANKODIA
AMPAD
SAMIYALA
Bombay
Mindi river
Mindi river
Viswamitri river
1
2
3
4
5
INDIA
Gujarat
GUJARAT
Baroda
National highway
Railway line
VUDA limit
Source of pollution
Observation station (Field survey)
1. GSFC + HWP
2. Oil refinery
3. IPCL
4. GACL
5. GIDC - Nandesari
1 Km
N

of sources of pollution. Six stations viz., Koyali, Undera, Dumad, Sankarda, Chhani and Ankodia were selected from medium pollution zone i.e. stations located at 3.5 to 6 Km., away from the major pollution source. One village, Ampad (14 Km., away from the pollution sources) was selected from low pollution zone. Control site, Samiyala is located 36 Km., away from the sources of pollution (Fig. 2.2.2.)

(ii) Morphological observations: The morphological observations on trees growing under natural conditions was carried out as per standard ecological methods (Misra, 1968). As it was difficult to ascertain the observations on same aged trees, circumference of the tree trunk at breast height (CBH) was considered as more reliable parameter for the comparative study. The observations were recorded on the trees with the following girth classes (CBH); Mango 125 to 150 Cms., Rayan 200 to 225 Cms. and Jamun 175 to 200 Cms.

Various methods are recommended to measure the height of the trees. Any instrument that can measure an angle of $45^{0}$ may be used (Michael, 1984). For this investigation simple height meter was used i.e. plastic triangle. The height of the tree was measured by looking from a point of height meter (the side measuring $45^{0}$) and walking backward or forward from the tree, until top of the tree, both ends of height meter and eye of the observer were in a straight line. Care was taken to keep the horizontal side

of the height meter always parallel to the ground. Distance from the tree to the observer and observer's height from the ground to the eye level were measured. The sum of these readings was taken as the height of the tree, though Saxton is used but it involves more calculations so this rapid method was used. Circumference of the trunk at breast height was recorded with measuring tape. Radius of the plant cover was measured in four directions from the trunk of the tree to determine canopy cover. Average of the four radii(r) was taken for calculating the canopy cover ( $\pi r^2$). The leaf area and leaf area damaged was measured with help of a transparent graph paper in the laboratory. Leaves were collected randomly from each tree. Mixture of the leaves of 10 trees and then 10% of the sample was taken for the study. Visual observations were made to express the percentage of leaflessness and flowering and then by comparing with control. To obtain the data on fruit yield, visual observations as well as data obtained from orchard owners and village panchayat officials were used.

(iii) Biochemical observations: Fresh, matured leaves collected from each station to determine different metabolites concentration. Leaf samples were collected in polythene bags and brought to the laboratory at the earliest possible in an ice box. Leaf samples were thoroughly washed under running water with a final rinse in distilled water.

B iochemical parameters studied were chlorophyll pigments a, b, protein, total soluble sugars, reducing sugars, total sulphur and chloride content. The extraction and estimation procedures are described in Chapter 2.3.3.

2.3.2. Soil study at different observation stations:

Soil samples collected from all the stations under investigation were brought to the laboratory, air dried, powdered with a mortar and pestle and sieved. Most of the parameters were analysed based on Jackson (1967) book.

Soil pH ( 1 soil : 1 water w/v ) and electrical conductance ( 1 soil : 5 water w/v) were determined using glass electrode digital pH meter and conductivity bridge respectively (Jackson, 1967).

The soil organic matter was determined by its oxidation at 400°C for 7 to 8 hours (Jackson, 1967).

Total nitrogen was determined by modified Kjeldahl method (Jackson, 1967). Soil samples were digested with concentrated $H_2SO_4$ with $CuSO_4$ $5H_2O$ and $K_2SO_4$ (1 :2) mixture as a catalyst. The digested samples were made upto required volume with glass distilled water and then distilled in microkjeldahl apparatus. The distillate collected in boric acid was titrated against standard HCl. The percentage nitrogen was calculated as follows:

$$\% \text{ N in soil} = (T - B) \times N \times \frac{104}{S}$$

Where T = Sample titration ( ml. std. acid)

B = Blank titration (ml. std. acid)

N = Normality of std. acid (HCl)

S = Sample weight (gms)

Soil sulphur content was determined colorimetrically after wet digestion of samples in acid mixture ( 3 $HNO_3$ : 2 Perchloric acid v/v ) as described by Garrido (1964).

Chloride content was determined in the soil solution by titrating against standard silver nitrate solution (Jackson, 1967).

2.3.3. Leaf anatomical study:

(i) Foliar epidermal study: Variation in foliar epidermal features due to pollution was investigated in the three fruit tree species under investigation. Highly polluted sites like Ranoli, Bajwa, Angadh and Omkarpura were selected for this study. These observations were compared with the control specimens collected from the University Botanical garden. Observations were recorded for number of stomata and epidermal cells per unit area, size of guard cells, stomatal aperature and density of trichome and abnormal stomata.

Leaf samples were collected during the morning hours ( 7 to 8 A.M.) from the field, fixed immediately in FAA

solution. Leaf bits were cut from the middle of the leaf (central portion between midrib and leaf margin) to avoid the border effect. Stomatal observations were made on the lower epidermis. Epidermal peelings were prepared mannually, stained with Toludine blue 'O' and observed in projected disc (Vesopan) attached with Carl-Zeiss microscope. Minimum 25 readings were recorded for each samples by random selection of microscopic fields. Stomatal index was calculated as per the following formula:

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

Where S = Number of stomata per unit area

E = Number of epidermal cells per unit area.

(ii) Fluorescence study: Chlorophyll fluorescence studies were done to determine the difference between polluted and control plants. Fine hand sections of fresh leaves were made and autofluorescence of chlorophyll was photographed by epifluorescence microscope (Zeiss) under 546 nm.

Foliar sections were stained with neutral red (Kirk 1970) and mounted on a clean glass slides. Cuticle was illuminated by fluorescene lamp. Secondary yellowish fluorescence of cuticle was photographed at 546 nm by epifluorescence microscope.

2.3.5. Field Exposure Study:

After careful observations of general damage on fruit trees, data on ambient air pollution level and wind direction a few experimental stations were established at Bajwa, Koyali, Omkarpura, Ranoli, Padamla, Sankarda, Fajalpur, Damapura and Angadh. University arboratum was kept as control. (Fig.2.2.3.).

One year old healthy mango, and jamun saplings obtained from the State Forest Department and three months old rayan saplings (raised from seeds in the University arboratum) were transplanted in well perforrated polythene bags (35 x 30 Cms) containing humus rich garden soil. After conditioning them in the arboratum for four weeks, were transported and placed in wire-net enclosures at aforesaid experimental stations in the pollution area and control in the University arboratum (least polluted area).

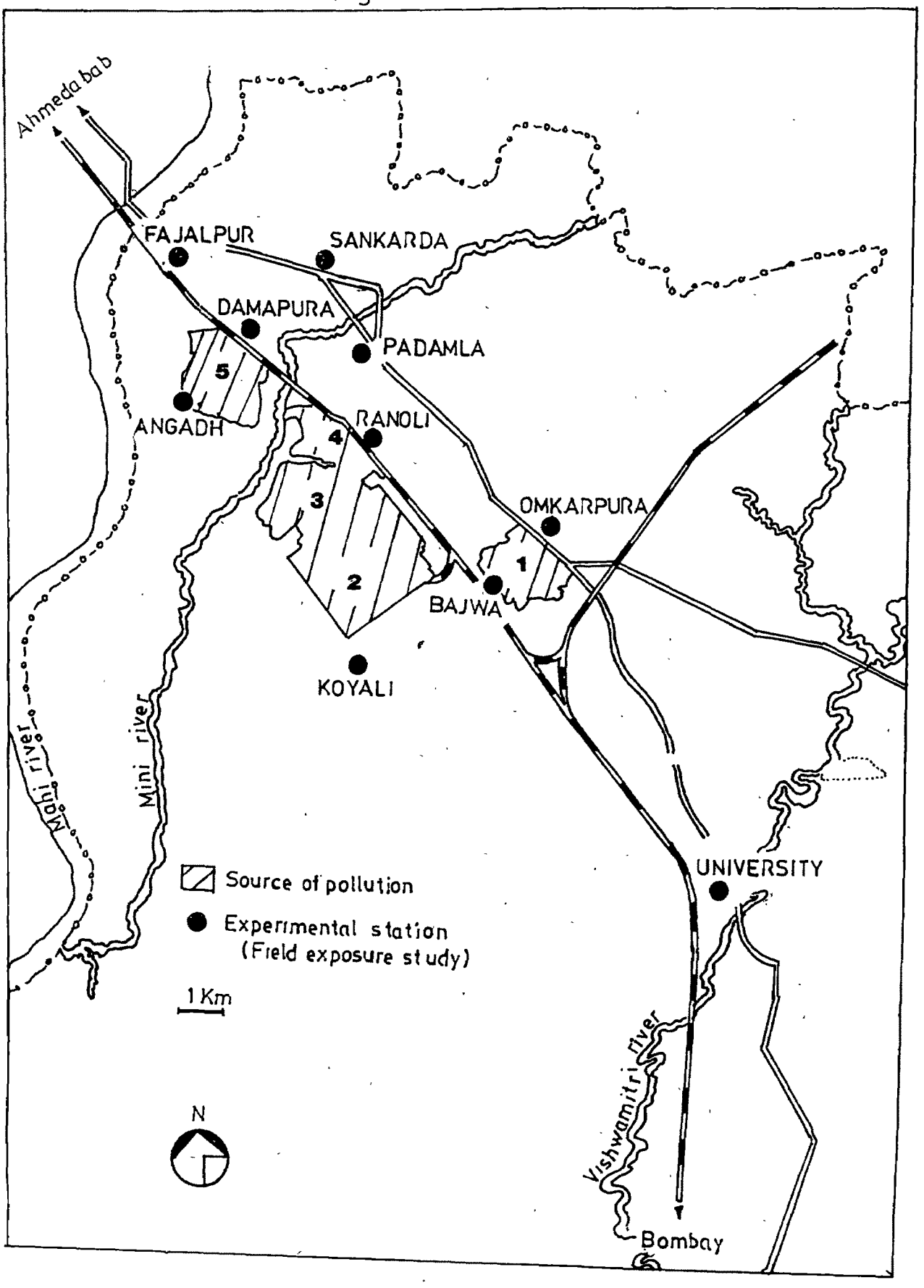
Ten replicates of each of jamun, rayan and mango saplings were placed for investigation, at all the experimental and control stations. All the saplings were examined at an interval of five days and watered from a common source (Science faculty). Other cultural practices were uniformly maintained for all the exposed saplings.

During summer season the plants at Fajalpur, Sankarda and Padamla were partially damaged by pathogens. So, those three

Fig.2.2.3. Map showing the location of different experimental stations for field exposure study

1. Gujarat State Fertilizer Company and Heavy Water Plant
2. Gujarat oil refinery
3. Indian Petrochemicals
4. Gujarat Alkalies and Chemicals Ltd.
5. Nandesari Industrial Estate

Fig. 2.2.3.


Ahmedabad
FAJALPUR
SANKARDA
DAMAPURA
PADAMLA
ANGADH
RANOLI
OMKARPURA
BAJWA
KOYALI
UNIVERSITY
Bombay
Mini river
Mahi river
Vishwamitri river
Source of pollution
Experimental station
(Field exposure study)
1 Km
N
1
2
3
4
5

stations were abandoned. During winter, mango and rayan saplings at Angadh and Bajwa respectively died due to acute pollution exposure. The study was continued for summer season with new set of mango and rayan saplings from garden at Angadh and Bajwa respectively.

The observations were recorded at monthly intervals and seasonal response was determined from the mean value. Mean values of the each season's (Monspon, Winter and Summer) observations are presented here.

Morphological parameters viz: shoot length, number of leaves/plant, total leaf area, number of branches/plant and percentage of leaves with symptoms were recorded. The injury index was calculated (Pawar, 1982) using the following formula:

$$\text{Injury index} = \frac{\text{Leaf area damaged}}{\text{Total leaf area of the plant}} \times 100$$

Foliar samples were analysed for the following biochemical parameters; chlorophyll pigments a, b , total chlorophyll, carotenoids, ascorbic acid, protein, total free aminoacids, total soluble sugars and sulphur content. The extraction and estimation procedures are described in the later part of this Chapter 2.3.7.

Data on wind direction and wind speed during the investigation period were obtained from Meteorological

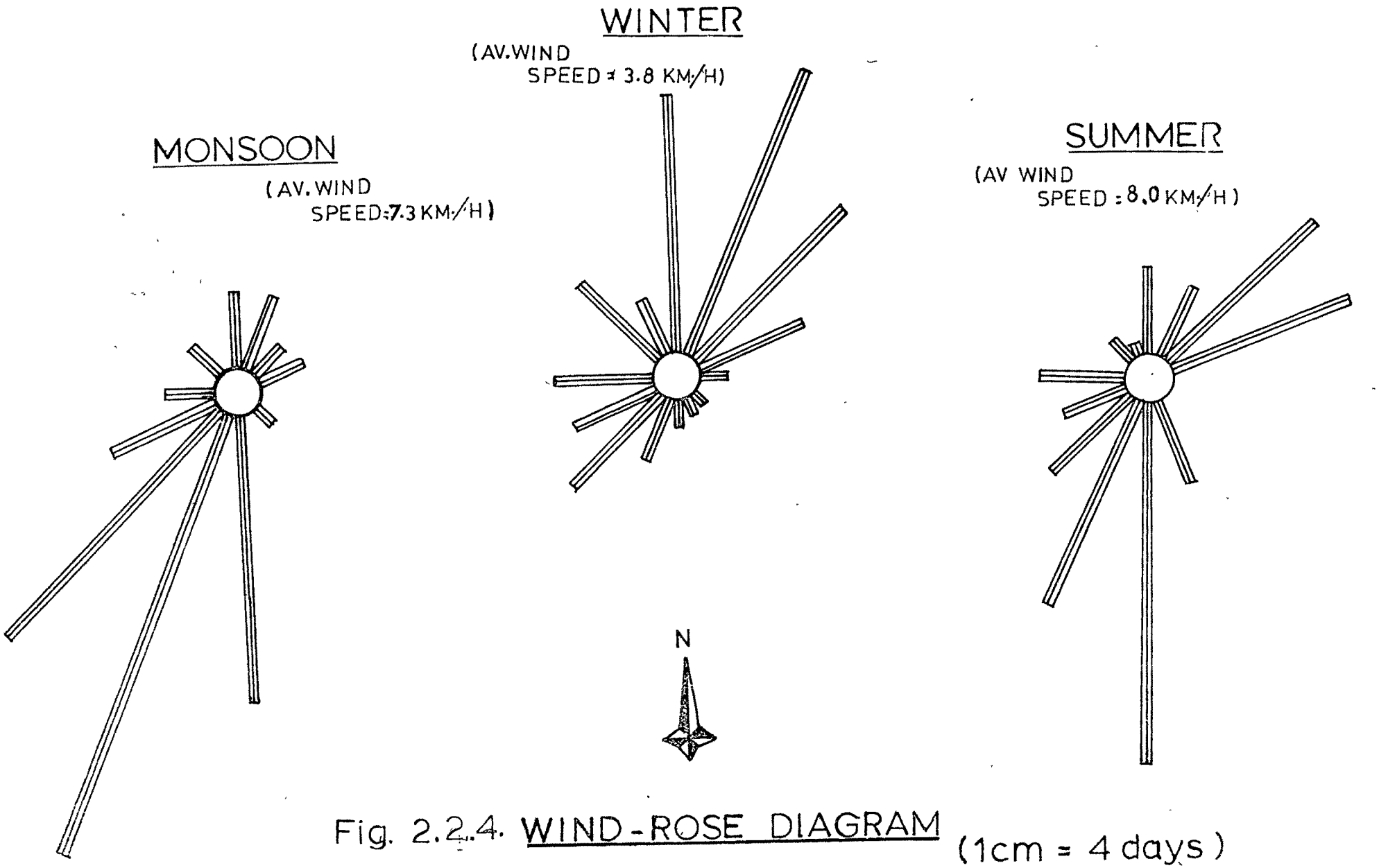



Fig. 2.2.4. WIND-ROSE DIAGRAM (1cm = 4 days)

department, The M.S. University of Baroda. The wind rose diagram for the three seasons (Monsoon, winter, and summer) was prepared from this data (Fig.2.2.4.).

2.3.6. Air pollution level in ambient air:

Ambient air quality data was collected from the study reports of National Institute of Occupational Health (NIOH). During this study period NIOH was monitoring air quality for VUDA at most of the observations stations. This was considered one of the main criteria for selecting the aforesaid observation stations for field survey and field exposure study.

The air pollutants monitored were sulphur dioxide ($SO_2$), Nitrogen oxides (NOx) and Suspended particulates matter (SPM). Continuous monitoring system was not possible. The samples were collected at an interval of six days. The sample collection for the gaseous pollutants ($SO_2$ and NOx), three samples representing morning, evening and night time ( 8 hourly basis) were collected, whereas for SPM single samples of 24 hours duration were collected. The well established and standard methods were used for collection and estimation of air pollutants. ( In absence of continous monitoring system, many peaks which might have caused heavy damage, were missed). Ambient air concentration of $SO_2$ (Table 6A), NOx (Table 6B) and SPM (Table 6C) during a season, annual arithmatic mean of daily average and frequency

of higher concentrations recorded are presented here.

Air pollution monitoring data of Nandesari was considered for Angadh and Damapura as these two stations were very close to Nandesari on its southwest and northeast directions respectively. So, the air monitoring data of Nandesari was considered close to these two stations. Monitoring data was not available for Undera, Dhanora, Chhani (Field survey), Koyali, Ranoli and Padamla (Field survey and Field exposure study).

Table 6.

Table 6. Air pollution level at different observation stations

A. Ambient air concentration of sulphur dioxide ($\mu g/m^3$)

| STATIONS | Seasonal average concentration | | | Annual arith. mean of daily average values | % observations greater than | | |
|---|---|---|---|---|---|---|---|
| | Summer | Monsoon | Winter | | 30 $\mu g/m^3$ | 80 $\mu g/m^3$ | 120 $\mu g/m^3$ |
| Bajwa | 9.6 | 5.6 | 38.5 | 17.9 | 11.5 | 0.8 | 0.1 |
| *Nandesari | 41.0 (126.6) | 30.3 (78.0) | 51.7 (138.4) | 26.2 | 31.9 | 17.0 | 8.5 |
| Omkarpura | 28.2 (126.3) | 21.5 (95.9) | 14.3 (85.6) | 21.3 | 11.8 | 0.8 | 0.8 |
| Dumad | 16.9 (86.0 | 8.4 (54.3) | 6.7 (39.8) | 10.7 | 2.5 | 1.0 | - |
| Fajalpur | 11.0 (56.0) | 9.1 (49.6) | 5.0 (28.3) | 8.4 | 1.7 | - | - |
| Ankodia | 6.3 (15.6) | 4.3 (20.9) | 12.2 (28.1) | 7.6 | - | - | - |
| Sankarda | 16 .4 (85.3) | 8.1 (59.9) | 10.3 (53.7) | 11.6 | 18.2 | 3.0 | - |
| Ampad | 7.9 (25.6) | 5.7 (19.9) | 6.4 (32.9) | 6.7 | 2.6 | - | - |
| Samiyala | 2.1 (16.8) | 3.6 (19.6) | 10.9 (35.3) | 5.6 | 4.2 | - | - |

( ) Maximum 8 hour average value

* Nandesari = Angadh and Damapura.

Table 6. Air pollution level at different observation stations.

B. Ambient air concentration of nitrogen oxides ($\mu g/m^3$)

| STATION | Seasonal average concentration | | | Annual arith. mean of daily average value | % Observations greather than | | |
|---|---|---|---|---|---|---|---|
| | Summer | Monsoon | Winter | | 30 $\mu g/m^3$ | 80 $\mu g/m^3$ | 120 $\mu g/m^3$ |
| Bajwa | 12.1 (71.7) | 32.4 (85.1) | 46.8 (134.6) | 30.4 | 23.0 | 4.8 | 1.3 |
| *Nandesari | 48.3 (96.4) | 35.8 (65.3) | 69.5 (142.9) | 51.2 | 31.9 | 17.0 | 8.5 |
| Omkarpura | 41.7 (137.4) | 61.5 (122.5) | 32.6 (96.3) | 45.3 | 37.8 | 14.1 | 6.7 |
| Dumad | 22.9 (49.3) | 28.5 (85.8) | 19.2 (66.1) | 23.5 | 26.0 | 2.0 | - |
| Fajalpur | 24.0 (56.0) | 34.7 (92.9) | 25.8 (89.1) | 28.2 | 22.1 | 3.9 | - |
| Ankodia | 15.9 (28.4) | 15.0 (38.8) | 20.9 (46.6) | 17.6 | 5.7 | - | - |
| Sankarda | 25.8 (125.3) | 28.4 (132.9) | 31.4 (106.9) | 8.6 | 48.7 | 23.0 | 10.8 |
| Ampad | 13.0 (48.1) | 22.9 (36.3) | 20.1 (56.1) | 18.7 | 9.5 | - | - |
| Samiyala | 12.7 (39.1) | 26.0 (34.7) | 29.7 (68.8) | 22.6 | 33.3 | - | - |

( ) Maximum 8 hour average value. * Nandesari = Angadh and Damapura.

Table 6. Air pollution level at different observation stations

C. Ambient air concentration of suspended particulate matter ($\mu g/m^3$)

| STATION | Seasonal average concentration | | | Annual arith. mean of daily average value | % of observations greater than | | |
|---|---|---|---|---|---|---|---|
| | Summer | Monsoon | Winter | | 100 $\mu g/m^3$ | 200 $\mu g/m^3$ | 500 $\mu g/m^3$ |
| Bajwa | 239 (330) | 213 (196) | 531 (715) | 202 | 50.5 | 24.9 | 6.9 |
| *Nandesari | 211 (397) | 186 (329) | 223 (428) | 207 | 64.2 | 7.1 | - |
| Omkarpura | 352 (462) | 272 (454) | 206 (363) | 310 | 75.7 | 32.4 | - |
| Dumad | 549 (1208) | 194 (530) | 237 (739) | 327 | 89.7 | 56.6 | 8.6 |
| Fajalpur | 278 (329) | 338 (535) | 216 (419) | 277 | 72.7 | 50.0 | 4.6 |
| Ankodia | 212 (463) | 139 (527) | 435 (846) | 262 | 89.0 | 26.0 | 7.1 |
| Sankarda | 352 (584) | 272 (461) | 322 (657) | 315 | 95.5 | 77.3 | 9.0 |
| Ampad | 212 (349) | 106 (367) | 277 (465) | 165 | 75.0 | 28.6 | - |
| Samiyala | 256 (452) | 156 (278) | 233 (383) | 215 | 85.7 | 57.1 | - |

( ) Maximum 8 hour average value. * Nandesari = Angadh and Damapura.

2.3.6. Artificial Fumigation study:

One year old healthy mango, rayan and jamun saplings grown in experimental pots having humus rich garden soil were used for the study. Five replicates for each set of study for the three tree saplings were exposed in the fumigation chamber in the following manner:

| | |
|---|---|
| Neither exposed to SO2 nor treated with ascorbic acid | = Control - 1 ($C_1$) |
| Exposed to $SO_2$ but untreated with ascorbic acid | = Control - 2 ($C_2$) |
| Exposed to $SO_2$ and treated with 10 µmoles of ascorbic acid | = Treatment -1 ($T_1$) |
| Exposed to $SO_2$ and treated with 100 µmoles of ascorbic acid | = Treatment - 2 ($T_2$) |

(i) Sulphur dioxide exposure:

$C_2$, $T_1$ and $T_2$ plants were exposed to 0.5 ppm of SO2 in a 1 $m^3$ acrylic fumigation chamber for two hours with two days interval upto 180 days (Table 7).

Plants were exposed to $SO_2$ by continuous flow method. $SO_2$ from cylinder (100%) was introduced into the chamber after diluting with atmospheric air by air blower and adjusting the air flow with rotameters. Dilution of $SO_2$ was adjusted with rotameters till the concentration of $SO_2$ within the chamber becomes 0.5 ppm. The SO2 concentration inside the chamber was periodically determined colorimetrically by absorbing the air from outlet of the

chamber in sodium tetra chloromercurate solution (West and Gaeke, 1956). Fan inside the chamber was switched on to facilitate uniform mixing of the gas. $C_1$ plants were also given similar treatment, but without $SO_2$. To minimize the temperature rise inside the chamber, exposures were carried out between 7.30 to 10.00 A.M. During exposure the temperature was increased by 0.5 to 1°C and RH by 2 to 3% inside the chamber than external atmosphere.

(ii) Ascorbic acid treatment and sampling:

Preliminary studies on the abatement of sulphur dioxide effect on plants with ascorbic acid was carried out. Two sets of $SO_2$ exposed plants were treated weekly with 10 µmoles ($T_1$) and 100 µmoles ($T_2$) separately. Plants were sprayed with ascorbic acid during the evening hours.

Biochemical changes in chlorophyll a, b and carotenoids, ascorbic acid, protein, total free aminoacids, total soluble sugars and sulphur content were determined in foliar tissues at an interval of 30 days.

Table 7. Number of $SO_2$ exposures and ascorbic acid treatments

| Sampling order | Cumulative $SO_2$ dose (Conc. in ppm x h x number of exposures = $ppmh^{-1}$) | No. of ascorbic acid treatment |
|---|---|---|
| I (30th day) | 0.5 x 2 x 10 = 10 | 4 |
| II (60th day) | 0.5 x 2 x 20 = 20 | 9 |
| III (90th day | 0.5 x 2 x 30 = 30 | 13 |
| IV (120th day) | 0.5 x 2 x 40 = 40 | 18 |
| V (150th day) | 0.5 x 2 x 50 = 50 | 22 |
| VI (180th day) | 0.5 x 2 x 60 = 60 | 27 |

2.3.7. Analytical methods of various biochemical parameters studied

(i) Chlorophyll pigments analysis:

Chlorophyll pigments were extracted from the fresh foliar tissues (Holden, 1965). 100 mg of fresh leaf samples was crushed in a pre-chilled mortar and pestle with cold 80% acetone. A pinch of calcium carbonate was added to avoid the phaeophytin formation. Glass powder was also added to quicken the processes. The final extract was centrifuged at 3000 rpm for 15 min. The supernatant was collected. This process was repeated for three times for complete extraction of pigments i.e. till the residue becomes colourless. The supernatant was made upto required volume. Care was taken to avoid direct exposure of extract to light during the

experiment to check chlorophyll degradation. OD of the extract was measured in the following nm in spectrophotometer; 480, 510, 645, 652 and 663.

Chlorophyll a, b and total chlorophyll contents were determined by Maclachlan and Zalik (1963) method.

$$\text{Chlorophyll } \underline{a} \text{ mg/g.f.wt.} = \frac{(12.3 \times OD\ 663) - (0.86 \times OD\ 645)}{d \times 1000 \times w} \times V$$

$$\text{Chlorophyll } \underline{b} \text{ mg/g.f.wt.} = \frac{(19.3 \times OD\ 645) - (3.6 \times OD\ 663)}{d \times 1000 \times w} \times V$$

$$\text{Total chlorophyll mg/g.f.wt.} = \frac{27.8 \times OD\ 652}{d \times 1000 \times w} \times v$$

Carotenoids content was determined by Duxbury and Yentsch (1956) formula:

$$\text{Carotenoids mg/g.f.wt.} = \frac{(7.6 \times OD\ 480) - (1.49 \times OD\ 510)}{d \times 1000 \times w} \times V$$

Where V = volume prepared (ml)

d = distance travelled by light (cm)

w = weight of the sample (gms)

(ii) Total ascorbic acid:

Ascorbic acid was extracted from fresh leaves with cold 5% metaphosphoric acid with 10% stannous chloride. Stannous chloride acts as a reducing agent to prevent the oxidation of ascorbic acid to dehydroascorbic acid

during extraction. Extraction was done in a cold atmosphere. Final extract was centrifuged at 3000 rpm for 5 min., and the supernatant was made upto known volume with 5% meta-phosphoric acid containing 0.5% $SnCl_2$. The filtrate was treated with activated charcoal. This converts the ascorbic acid into dehydroascorbic acid which was determined colori-metrically by reacting with 2-4, Dinitro phenyl hydrazine (Schafhest and Kingslay, 1955). A standard graph was plotted using AR grade ascorbic acid.

(iii) Protein content:

200 mg of fresh leaf sample was grounded in 0.01 M Na-K-phosphate buffer, pH 7.6, containing 0.1M NaCl. The ratio of tissues to grinding medium was 1 : 1 (w/v) . The homogenate was centrifuged at 3000 rpm for 10 min. An equal volume of 10% Trichloroacetic acid was added to this supernatant. After centrifugation, the pellet was re-suspended in a volume of 0.1N NaOH equal to the initial supernatant and the protein content was determined by Hartee's (1972) method. Crystalline Bovine albumin was used as standard protein source.

(iv) Total free aminoacids:

100 mg of powdered leaf sample was suspended in 4 ml of methanol: chloroform : water mixture (MCW 12:5:3 v/v) and centrifuged. The supernatant was collected and the residue was reextracted by shaking it for 5 min., with further addition of 4 ml NCW. The mixture was centrifuged and the second

supernatant was added to the first, then the tissue extract was further extracted four times in the same way with 80% ethanol. All the extracts were combined and dried at 35°C. The dried extract was dissolved in water and used directly for total free aminoacids estimation by ninhydrin method (Rosen, 1957).

(v) Total soluble and reducing sugars:

Extraction of soluble and reducing sugars was carried out with 80% ethanol, according to the procedure of Mc Cready et al., 1950 . Dry powder of 100 mg leaf sample was taken in a centrifuge tube. 5 ml. of boiling 80% ethanol and 5 ml of distilled water were added and stirred well. The samples were centrifuged at 5000 rpm for 15 min. Extraction was repeated five times. After cooling, the supernatant was separated by sedimentation. Supernatant was collected in an evaporation dish and aliquot was evaporated to near dryness at 60°C. The residue was dissolved in a warm water and filtered through glass wool. The volume was made upto the required volume. This solution was used for determination of total soluble and reducing sugars.

Assay of soluble sugars: Three ml of the sample and 6ml of anthrone reagent (prepared freshly by dissolving 0.2gm. of anthrone in 100 ml of 95% $H_2SO_4$) was thoroughly mixed and placed in a boiling water bath for 3 min. Then it was cooled in ice. The colour developed was read at 620 nm (Yemm and

Willis, 1954) & the OD was compared with the standard curve prepared with glucose.

Assay of reducing sugars: To the 4ml of sample, two ml of 0.4% potassium ferricyanide (prepared freshly by dissolving 4g of potassium ferricynide in 100 ml of distilled water) and 1 ml of carbonate cyanide (stock solution prepared by dissolving 0.1500 gm., of sodium cyanide and 8 gm. of anhydrous sodium carbonate in 500 ml of distilled water) were added. The sample was mixed thoroughly and heated for 8 min., in a boiling water bath. After cooling for 2 minutes at room temperature 5 ml of ferric iron ( stock solution prepared by suspending 20 gm . of gumghatti in one litre of water in a cheese cloth for 24 hours. To the solution a mixture of 5 gm. of anhydrous ferric sulphate + 75 ml of 85% phosphoric acid and 100 ml of water was added, drop by drop 1% $KMnO_4$ solution was added until a slight pink colour developed) was added to provide colour. The volume was made upto 25 ml with distilled water and was read at 520 nm. The OD was compared with standard curve prepared with glucose.

(vi) Total sulphur content:

Total sulphur content in the dry foliar powder was determined colorimetrically after wet digestion in acid mixture of concentrated $HNO_3$ and 70% perchloric acid (3 : 2 v/v)(Garrido, 1964).

(vii) Chloride content:

Foliar chloride content was determined titrimetrically (Humphries , 1956). Dry leaf samples were ashed at 500° C in the presence of calcium oxide to prevent loss of chlorine. Ash solution with standard silver nitrate solution was back titrated against the standard ammonium thiocyanate. The chloride content was determined based on the following equation:

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